THE UNIVERSITY *of* EDINBURGH

The effect of hypertension on the structural and functional integrity of the young and aged brain in an inducible transgenic model

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Declaration

I declare that this thesis comprises of my own work and has not been submitted previously as part of any degree. The work within this thesis was carried out by myself, unless where acknowledged in the text and all sources of data and information are referenced.

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Abstract

Hypertension has been associated with causing deleterious effects to the cerebrovasculature, which are thought to underlie the formation of white matter lesions (WML) and predispose individuals to age related cognitive decline. In humans hypertension frequently occurs concomitantly with other vascular risk factors making it difficult to ascertain the primary mechanisms of hypertension in isolation. Animal models of hypertension have been used in an aid to establish the mechanisms of hypertension in isolation. To date the knowledge gleaned from animal models has undoubtedly provided an insight as to the role of hypertension and cerebrovasculature remodelling but, these models have limitations such as lack of genetically matched controls and the inability to control the severity of hypertension, restricting the understanding of the underlying mechanisms. All studies within this thesis used the Cyp1a1 Ren2 inducible hypertensive rat model, induced by dietary addition of Indole-3-carbinol (I3C), allowing the severity and duration of hypertension to be tightly controlled and compared to genetically matched controls. This thesis set out to address the hypothesis that sustained hypertension will lead to alterations to the structural integrity of the cerebrovasculature and white matter, which will be exacerbated with age and that hypertension will be associated with alterations to gene expression and cognitive function.

Initially this thesis sought to investigate the effect of hypertension on the structural integrity of the vasculature in the Cyp1a1 Ren2 rat model. Firstly, blood pressure in the Cyp1a1 Ren2 rat model was characterised and it was found that the

dietary addition of I3C, caused a sustained level of increased blood pressure in all three cohorts.

Cerebrovascular alterations were found to consist of increased eNOS expression in the young brain, which progressed with increased duration of hypertension to vascular morphological alterations of decreased vessel width and a redistribution of tight junction protein claudin-5. With age, hypertensive vascular alterations consisted of increased eNOS expression and vascular density. Additionally, there was evidence that hypertension caused a vascular inflammatory response in the young and aged brain.

Secondly, this thesis investigated the effect of hypertension on gene expression. Overall it was found that hypertension altered genes related to collagen growth factors, ion channels, eNOS related Map-Kinase pathway and inflammatory genes.

Thirdly, this thesis sought to investigate the impact of hypertension on the overall structural integrity of the brain and white matter examining neurons, myelin, oligodendrocytes, axons and microglia, in several regions of the young and aged brain. In general, this study found that hypertension did not cause overt structural or myelin alterations in the majority of regions analysed, with only evidence of myelin alterations occurring within the subcortex of hypertensive animals from each of the young cohorts analysed. However, an adverse subcortical inflammatory response was found in hypertensive animals of the young 6-month cohort and also in hypertensive animals from the aged 4-month cohort, where the inflammatory response was not

exclusive to the subcortex of hypertensive animals but also occurred in multiple white matter tracts.

Lastly this thesis chose to examine the effect of hypertension on cognitive function, specifically spatial reference and working memory using the Morris water maze and found no evidence of alterations in the cognitive functions examined.

Conclusions

The results presented within this thesis demonstrated that hypertension in isolation leads to modest alterations to the integrity of the cerebrovasculature and white matter, with no evidence of alterations to specific cognitive functions examined, demonstrating the importance of studying hypertension in isolation. Additionally, this study highlights the initial hypertensive induced alterations to the cerebrovasculature, such as endothelial signalling, vascular structure and inflammation, providing a window for therapeutic intervention at a time point when there are minimal alterations to the overall structural integrity of the brain. Future studies in this model should concentrate on examining different severities of hypertension and also hypertension concomitantly with other vascular risk factors to try and recapitulate pathological alterations found in humans.

List of abbreviations

ACA	Anterior cerebral artery
ACE	Angiotensin converting enzyme
ACOA	Anterior communicating artery
AGES	Age, gene/Environment Susceptibility Reykjavik study
AICA	Anterior inferior cerebral artery
AKT	Protein kinase B
ANGII	Angiotension II infusion
ANOVA	Analysis of variance
APP	Amyloid precursor protein
ARIC	Atherosclerosis Risk in the Communities study
ARIC	Atherosclerosis risk in communities study
ASA	Anterior spinal artery
BA	Basilar artery
BBB	Blood-brain barrier
CBF	Cerebral blood flow
CC1	Anti-adenomatous polyposis coli protein
cDNA	Complementary DNA
cGMP	Cyclic guanosine monophosphate
CHS	Cardiovascular Health study
CNS	Central nervous system
CRP	C-reactive protein
CT	Computed tomography
Cyp1a1	Cytochrome P450 1a1
DAB	Diaminobenzidine
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular-signalling related kinase ½
ESAM	Endothelial cell-selective adhesion molecule
Et-1	Endothelin-1
EVA	Epidemiology of vascular aging

FDR	False discovery rate
Fgf	Fibroblast growth factor
FHS	Framingham Heart study
GFAP	Glial fibrillary acidic protein
H&E	Haematoxylin and eosin
HRP	Horseradish peroxidase
I3C	Indole-3-carbinol
Iba1	Ionized calcium-binding adapter molecule 1
ICA	Internal carotid artery
ICAM-1	Intercellular adhesion molecule-1
IGF	Insulin growth factor
IGFBP	Insulin growth factor binding protein
IL-6	Interleukin-6
JAM	Junctional adhesion molecule
JNK	c-Jun NH(2)-terminal kinase
JNK	c-Jun N-terminal kinase
MAG	Myelin associated glycoprotein
MAGUK	Membrane associated guanylate kinases
MAP	Mean arterial pressure
MAP-kinase	Mitogen-activated protein-kinase
MBP	Myelin basic protein
MCA	Middle cerebral artery
MCP-1	Monocyte chemotactic protein -1
MES	2-(N-morpholino) ethanesulfonic acid
MRI	Magnetic resonance imaging
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHS	National health service
NO	Nitric oxide
PAS	Periodic acid Schiff's
PBS	Phosphate buffered saline
PCA	Posterior cerebral artery
PCA	Principal component analysis
PCOA	Posterior communicating artery
PECAM	Platelet endothelial cell adhesion molecule
PI3K	Phosphatidylinositol 3-kinase-dependent
PI3K	Phosphoinositide 3- kinase
PROGRESS	Perindopril protection against recurrent stroke study (PROGRESS)
PVDF	Polyvinylidene fluoride
RAAS	Renin-angiotensin-aldosterone system
rCBF	Regional cerebral blood flow
RES	Rotterdam Extension Study
RIN	RNA integrity number
RMA	Robust multichip average

RNA	Ribonucleic acid
ROD	Relative optical density
ROS	Reactive oxygen species
RS	Rotterdam Study
SCA	Superior cerebral artery
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide Gel electrophoresis
sEH	Epoxide hydrolase
SHR	Spontaneously hypertensive rat
SHRSP	Spontaneously hypertensive rat stroke prone
SMA	α-smooth muscle actin
SVD	Small vessel disease
TGF-b	Transforming growth factor beta
TNF-α	Serum ameloid type A, tumour necrosis factor alpha
TrkB	Brain derived neutrophic factor receptor
VA	Vertebral artery
VEGF	Vascular endothelial growth factor
WAIS	Wechsler adult intelligence scale
WKY	Wistar Kyoto
WM	White matter
WML	White matter lesions
ZO	Zona occludens

1. Introduction

The structural and functional integrity of the brain requires a continuous, uninterrupted supply of energy (oxygen and glucose), through circulating blood flow. Under normal conditions, cerebral blood flow (CBF) must be sufficient to meet the energy demands imposed by neuronal activity within that region. This co-ordination of supply and demand is made possible by the close association of neurons, glia and vascular cells forming the conceptual 'neurovascular unit' (Fig 1.1) (del Zoppo and Mabuchi, 2003; Woolsey et al., 1996). In humans, hypertension is associated with causing deleterious actions to the structural integrity of the cerebrovasculature, which is thought to lead to a de-harmonistion between neuronal demand and vascular supply. This de-harmonisation is thought to underly gross structural and function alterations, which are often found in the aged brain, making hypertension a risk factor for the formation of white matter lesions and cognitive decline (Iadecola and Davisson, 2008). However, in humans hypertension is frequently present concurrently with other metabolically linked vascular risk factors, with less than 20% occurring in isolation (Kannel, 2000). Therefore, animal models are required to investigate the mechanisms of hypertension in isolation. Studies contained within this thesis sought to investigate hypertensive induced structural and functional alterations to the brain using the Cyp1a1 Ren2 inducible hypertension animal model, in which comparisons are drawn from genetically matched litter-mate control animals with onset, duration and severity of hypertension tightly controlled.

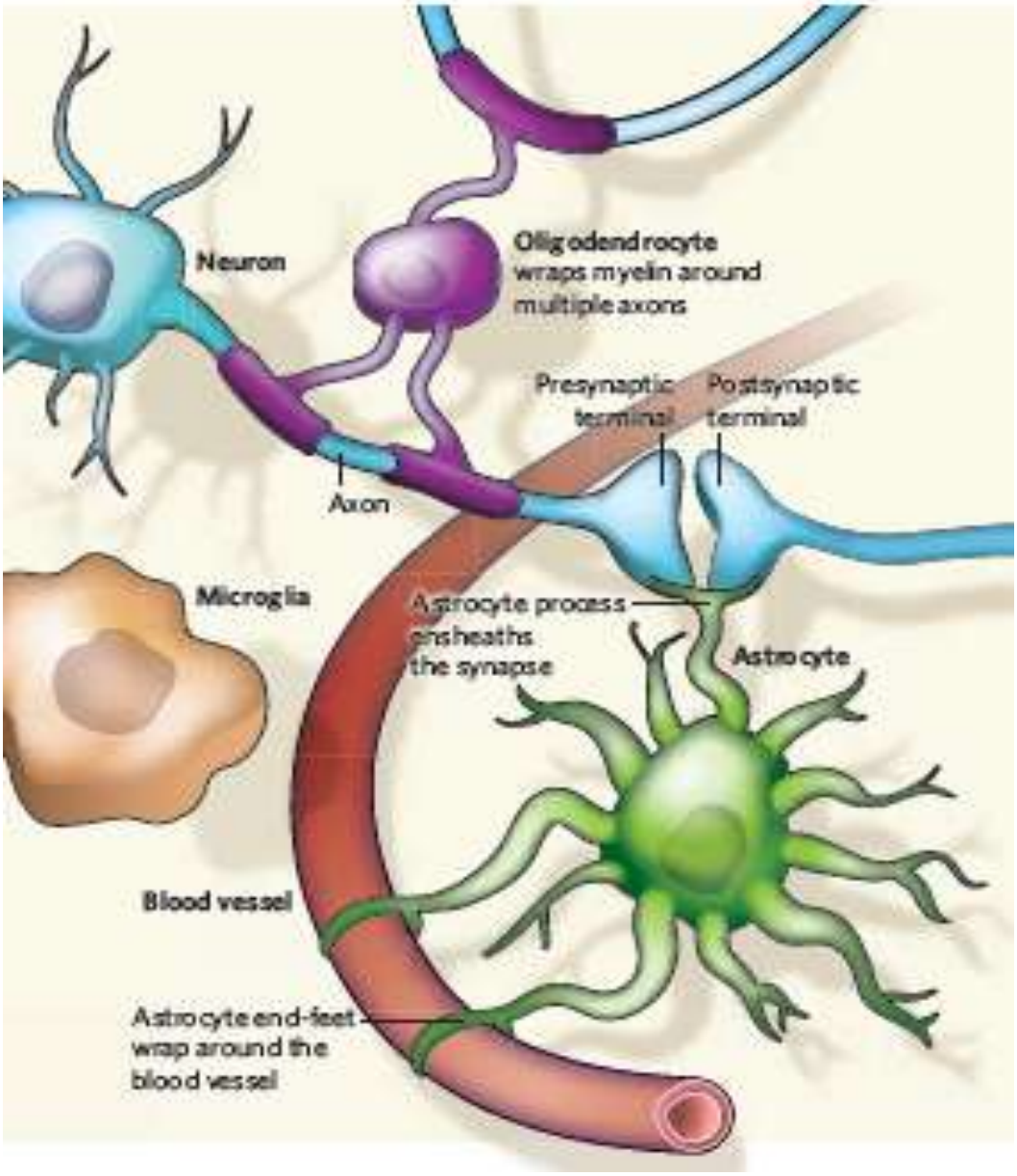

Figure 1.1: Components of the neurovascular unit

The neurovascular unit is a conceptual unit comprising of neurons, glia and blood vessels. The grey matter of the brain is made up of cell bodies and the white matter consists of oligodendrocytes, astroglia, microglia and axons. The capillaries form the cerebrovascular network supplying nutrients to the brain and the close association with neurons allows for energy demands to be met. (Image adapted from Allen & Barres 2009).

1.1. Hypertension

Hypertension is a substantial cause of premature morbidity and mortality within the UK, accounting for more than £1 billion in National Health Service (NHS) pharmaceutical costs alone (National Guideline Centre, 2011). Clinically hypertension is a chronic elevation of blood pressure, above the normal range of ≥140/90mmHg, commonly of unknown aetiology. Demographically, hypertension affects a quarter of the UK adult population, with more than half of those over the age of 60, showing a strong prevalence towards the ageing population (Fig 1.2) (Krause et al., 2011).

1.1.1. Risk factors for the development of hypertension

The cause of hypertension is commonly unknown, being attributed to a combination of environmental and genetic factors (Messerli et al., 2007; Staessen et al., 2003). Along with age, and in part gender, the occurrence of hypertension is also influenced by environmental lifestyle factors most of which are preventable, such as: smoking, poor diet, lack of exercise, type 2 diabetes and obesity (Fig 1.3) (Madhukumar and Gaikwad, 2012; Staessen et al., 2003). A genetic factor has also been identified with hypertension, with the majority of genes identified forming functional pathways concerning the renin-angiotensin-aldosterone and kallikrein-kinin system (two systems controlling blood pressure, fluid retention and coagulation) and also several ion transport genes (Kraja et al., 2011; Weder, 2007). The combination of environmental and genetic factors is the basis for the treatment of hypertension, with patients advised to undertake lifestyle alterations and pharmacological treatments commonly targeting components of the renin-

angiotensin-aldosterone system such as angiotensin II inhibitors (Higgins et al., 2007), aimed at lowering blood pressure prior to structural and functional alterations.

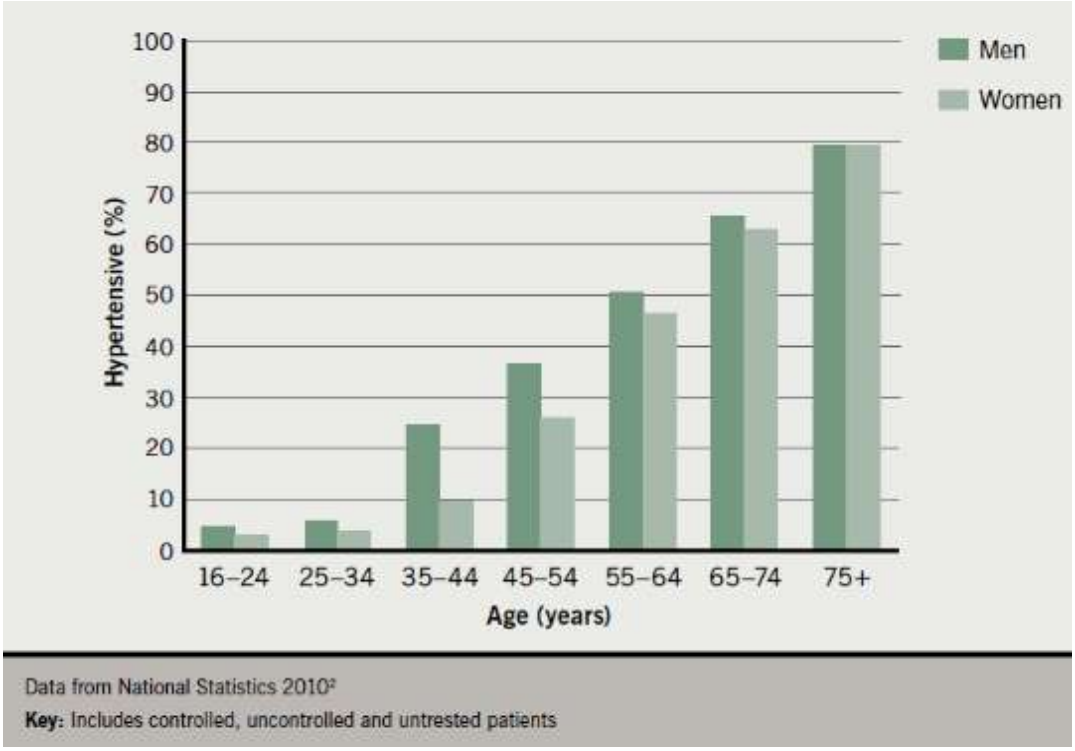

Figure 1.2: Prevalence of hypertension in men and women ranked by aged.
In 2010, UK national statistics found that hypertension (classified as those with blood pressure readings of ≥ 140/80 mmHg or those taking anti-hypertensive medication), increased in prevalence with age regardless of gender, with more than 2/3$^{rd's}$ of the population over the age of 65 reported as hypertensive or taking hypertensive treatment(s). (Image from (McCormack et al., 2013))

Risk factors		Total no. of Individuals	Hypertension (%)	O R (C I)
Sex	Male	845	81 (9.6%)	1.63 (1-2.47)
	Females	656	40 (6.09%)	
BMI	BMI >25	210	76 (36%)	15.70 (10.23-24.16)
	BMI<25	1291	45 (3.5%)	
Family History	Yes	264	74 (28%)	9.86 (6.52-14.95)
	No	1237	47 (3.8%)	
Additional salt intake	Yes	92	29 (31%)	6. 59 (3.93-11.02)
	No	1409	92 (6.5%)	
Diet	Non Veg	868	76 (8.8%)	1.2 (1-1.87)
	Veg	633	45 (7.1%)	
Smoking	Yes	276	86 (31.15%)	15.39 (9.90-24)
	No	1225	35 (2.9%)	
Alcohol	Yes	213	83 (38.96%)	21 (13.4-32.85)
	No	1288	38 (2.95%)	

Table 1.1: Risk factors for the development of hypertension

Hypertension is associated with multiple risk factors, namely a mixture of genetic and lifestyle dependent. This multifactorial nature of hypertension is one of the complications in understanding the mechanisms of hypertension in isolation in humans. BMI (body mass index) was calculate by dividing the subject mass over height and grouped into those with a BMI of more or less than 25. Family history was characterised by a positive or negative history of hypertension. Additional salt intake was characterised as more than two pinches of salt in each meal. Smoking was defined as yes or no and alcohol intake was define as yes for regular alcohol consumption and no for no alcohol consumption/occasional consumption (Image from Madhukamar et al 2012).

1.2. Cerebral blood flow

1.2.1. Cerebral blood flow in the normal brain

The human brain occupies around 2% of the total body mass, but demands ~20% of the total cardiac output for normal brain function, due to a lack of energy stores and high energy requirements (Sokoloff, 1989). Under normal physiological conditions, CBF is regulated by the brains metabolic requirements imposed by neuronal activity. Thus, during periods of increased neuronal activity, cerebral blood vessels are able to increase blood flow to regions of higher activity, a process known as functional hyperaemia (Cox et al., 1993; Ngai et al., 1988; Silva et al., 2000). Evidence suggests that there is a co-ordinated signalling between neurons, astrocytes and vascular cells during activity leading to an increased vasodilatory response. These properties of the vasculature and the close proximity with surrounding cells, endow the brain with hemodynamic control mechanisms, ensuring adequate CBF during fluctuations in activity (Astrup et al., 2008; Iadecola and Nedergaard, 2007; Niwa et al., 2001).

These hemodynamic control mechanisms also ensure that CBF is maintained at a constant level during alterations in blood pressure, by a combination of cerebral perfusion and vascular resistance. The cerebrovasculature is able to adapt to fluctuations in perfusion pressure by vasoactive responses, ensuring vasoconstriction in response to increased pressure and vasodilation in response to decreased pressure. This is known as cerebral autoregulation, which maintains constant blood flow within arterial pressures of 60-150mmHg during normal daily fluctuations (Paulson et al., 1990). This vasoactive response to pressure change is reliant on myogenic,

neurogenic, endothelial, metabolic and hormonal derived mechanisms (Farkas and Luiten, 2001). In turn, other vasoactive factors include ions, which can be induced by action potentials and synaptic transmissions (Drake and Iadecola, 2007). The existence of these above mechanism is a critical component in maintaining the homeostasis of normal brain function and structural integrity.

1.2.2.1. Cerebral blood flow in the hypertensive brain

Hypertension has been associated with leading to alterations in CBF by disrupting cerebrovascular control mechanisms. Paulson et al., found cerebral autoregulation was altered in chronic hypertensives, with a shift towards higher blood pressures, resulting in higher pressures required to maintain adequate blood flow. These alterations were postulated to be due to altered vasoreactivity, which may expose hypertensive patients to periods of decreased CBF (Paulson et al., 1990). In addition, hypertension appears to target specific regions of the brain as described by Fujishima et al., providing evidence of decreased CBF and oxygen metabolism within in the supratentorial regions: cortex, striatum and thalamus (Fujishima et al., 1995a). Additionally, a 7 year longitudinal study examining rCBF in aged hypertensive and normotensive individuals, found hypertensives to have decreased CBF in the prefrontal, anterior cingulated, occipital areas and also in the thalamus and cerebellum (Beason-Held et al., 2007). These findings suggest that hypertension primarily targets regions supplied by the perforating penetrating arterioles.

Moreover hypertension has also been associated with alterations to functional hyperaemia. Jennings et al., found that during cognitive testing normotensive individuals had increased CBF within the active regions but this was dampened in

hypertensive individuals in the posterior parietal area, middle posterior arterial watershed region and thalamus, which impacted negatively on cognitive function (Jennings et al., 2005). Overall, there is evidence that hypertension may influence CBF, which could impair the structural and functional integrity of the brain.

1.3. Normal brain structure

The human brain is not homologous, rather it was discovered in the early eighteenth century to be divided into two distinct parts: the grey and white matter (Clarke and O'Malley, 1996), with the WM found to occupy over half of the brain (Fields, 2008). The WM contains oligodendrocytes, astrocytes and microglia but, unlike the grey matter is devoid on neuronal cell bodies. It was established that the brain's WM tracts contain the axons extending from neurons, with the axons forming part of the connection between the brains white and grey matter, facilitating the flow of electrical signals by synaptic transmission throughout the brain over long distances (Clarke and O'Malley, 1996). In turn, normal brain function is made possible by the close association of neurons, vascular cells and glia, forming the conceptual 'neurovascular unit', allowing the microcirculation to be highly responsive to the energy demands within each region of the brain (del Zoppo and Mabuchi, 2003; Woolsey et al., 1996).

1.3.1. Neurons

Neurons form the signalling unit of neurotransmission and are highly sensitive to changes in their environment, with the ability to regulate both their internal and external electrolyte concentration, preventing the entry of toxic calcium into the cell, whilst the cell membrane is able to transmit electrical signals. This

allows neurons to encode and transmit information via action potentials throughout the brain (del Zoppo, 2006). Notably, it was discovered that there is a form of hemodynamic coupling between neurons and the cerebrovasculature, mediating CBF to sufficient levels for neuronal activity (Buxton et al., 2004). In turn, the close association of neurons with glia allows for sufficient neuronal transmission by neurotransmitter trafficking between neurons and glia, forming a bridge between the demands of neurons and the delivery of sufficient energy by vascular cells (Haydon and Carmignoto, 2006; Zonta et al., 2002).

1.3.2. Cerebrovascular structure

Higher mammals are endowed with a cerebrovascular structure designed to maintain CBF uniformly throughout the brain. Blood is supplied through the internal carotid arteries supplying the forebrain, the basilar artery and the hind brain. These arteries feed into the circle of Willis and due to its circular structure allow for collateral circulation; in the event of occlusion to one artery, more distal arteries can receive their blood supply from another artery (Liebeskind, 2003). The cerebral blood vessels arise from the Circle of Willis, branching into smaller arteries and arterioles, penetrating from the surface of the brain deep into the brain's substance and differ in cellular constituents with size in occurrence with their temporal location (Fig 1.4) (Drake and Iadecola, 2007). These surface arteries are collectively termed, the pial arteries (Jones, 1970) and contain a layer of both endothelial and smooth muscle cells, surrounded by an outer layer of leptomeningeal cells (collagen and fibroblasts) and perivascular nerves arising from the autonomic and sensory ganglia (Hamel, 2006; Peters, 1991). The vascular wall of the pial arteries is separated from the brain by a fluid filled space known as the Virchow-Robin space (Peters, 1991).

As pial arteries and arterioles penetrate deeper into the substance of the brain, they retain their same cellular composition, but are contacted by the end-feet of the astrocytic processes, forming a glial limitans membrane on the outermost side of the Virchow-Robin space (Takano et al., 2006). As these vessels penetrate even deeper into the brain, there is a progressive loss of the Virchow-Robin space and smooth muscle layers. These vessels are known as intracerebral arterioles, which branch to produce smaller arterioles and capillaries, whose density is regionally heterogeneous and activity dependent. These vessels have astrocytic end-feet and neural processes in primary contact with the basal lamina (Girouard and Iadecola, 2006). In the capillaries, smooth muscle cells are replaced by pericytes, which have many similar characteristics to smooth muscle cells and are able to modulate capillary diameter in response to stimulation (Cohen et al., 1996; Rennels and Nelson, 1975). These pericytes form processes which wrap around the endothelium, contacting endothelial cells through distinct 'peg like socket' contacts, and are an essential component in microvascular stability and function (Von Tell et al., 2006). These small penetrating arterioles and capillaries constitute the vascular supply to the deep subcortical WM regions of the brain, branching off at right angles, producing short non-furcating capillaries with few anatosomes. This leaves the deep subcortical WM regions of the brain particularly sensitive to alterations in blood flow, due to a lack of collateral circulation (De Reuck, 1971; Pantoni and Garcia, 1997b).

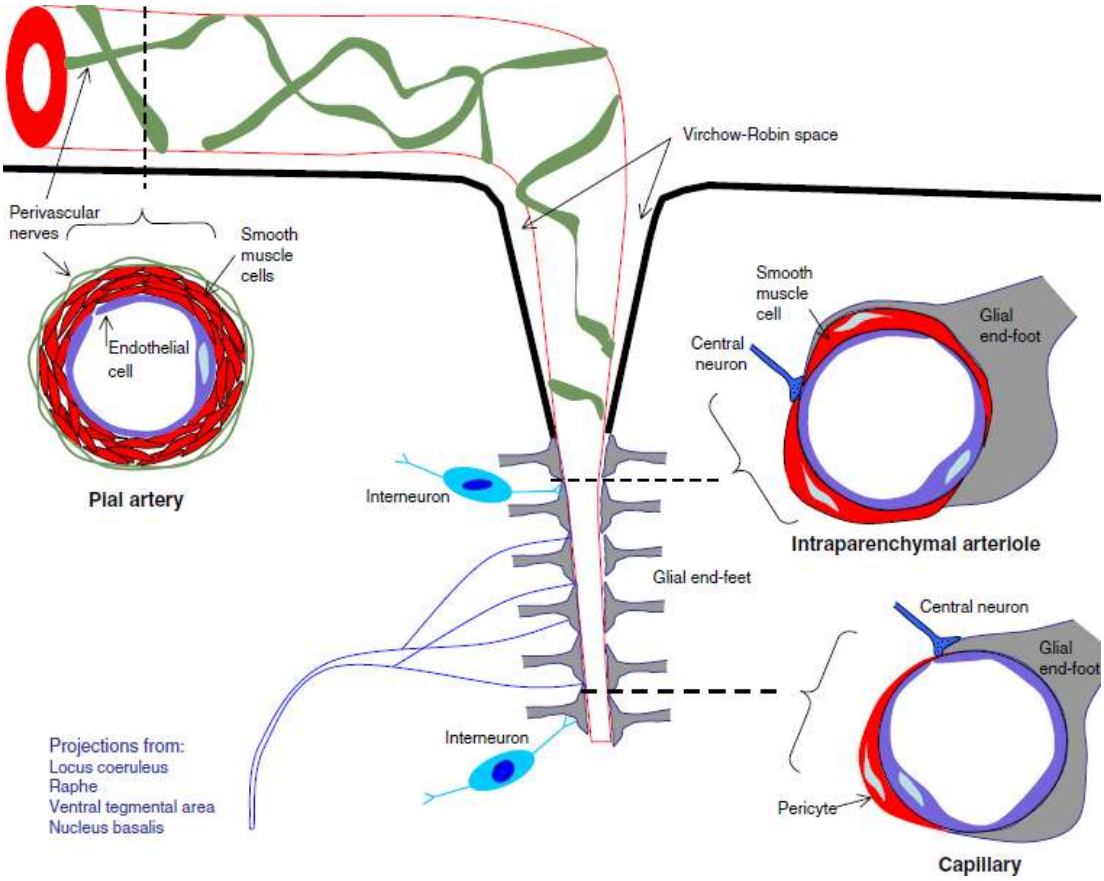

Figure 1.3: Cerebrovascular structure within the brain

As vessels penetrate into the substance of the brain, they branch off and differentiate from those vessels forming the circle of Willis. As vessels penetrate deeper into the brain, there is a progressive loss of the Vinchrow-Robin space and smooth muscle cell layer, which is eventually replaced by pericytes in the smallest most deeply penetrating vessels, the capillaries. (Image adapted from Drake & Iadecola., 2007).

1.3.2.1. The endothelial blood-brain barrier

Along with energy supply the cerebrovasculature is also equipped with the crucial role of preventing the entry of harmful stimuli into the brain, maintaining the neuroparenchymal microenvironment, by the formation of the endothelial blood-brain barrier (BBB), allowing effective neuronal function, ensuring concentrations of ions are maintained within narrow limits (Hodgkin 1951). The endothelial BBB was firstly discovered by Paul Ehrlich who observed that the injection of water soluble dyes into the circulatory system stained all organs apart from the spinal cord and the brain (Ehrlich 1885). Following studies using high resolution EM found that the capillary endothelial cells within the cerebral vasculature were bridged by tight junctions and were responsible for the establishment of an impermeable membrane and the anatomical substrate of the BBB (Reese and Karnovsky 1967 and Brightman). Endothelial cells forming the BBB are distinct from those found within the periphery by lack of fenestration (Febstermacher 1988), low pinocytotic activity (Sedlakova 1999), high mitochondrial content (Oldendorf 1977) and the presence of tight junctions (Kniesel and Wolburgh 2000).

The function of the endothelial BBB is primarily to limit paracellular flow of ions and harmful stimuli, serving as the brain's protective filter (Fig 1.5) (Begley et al., 1990; Risau and Wolburg, 1990). Hydrophobic molecules can cross the BBB, across the endothelium by transcellular mechanisms of carrier mediated transport, ion transport, active efflux transport, receptor mediated transport and caveolae mediated transport (Zlokovic, 2008). These endothelial cells lining the vessels form a chain of cells and as described above are connected by tight junctions (Kniesel and Wolburg, 2000; Vorbrodt and Dobrogowska, 2003; Wolburg and Lippoldt, 2002),

but there are also other junctional proteins connecting the endothelial cells, forming the endothelial BBB namely: adheren (Schulze and Firth, 1993) and gap junctions (Fig 1.5) (Braet et al., 2001; Kojima et al., 2003; Simard et al., 2003; Tao-Cheng et al., 1987). These linking junctions have specific roles within the vasculature, with tight junctions and adheren junctions mainly limiting permeability across the BBB, whereas gap junctions are thought to participate in intercellular communication (Bazzoni and Dejana, 2004).

1.3.2.2. Endothelial tight junctions

Tight junctions of the endothelium are proposed to be the key structures responsible for barrier properties (Brightman and Reese, 1969; Kniesel and Wolburg, 2000; Mollgard and Saunders, 1986; Nabeshima et al., 1975). Freeze fracture studies identified tight junctions as intra-membranous networks of multiple strands forming occluded intracellular clefts (Brightman and Reese, 1969; Farquhar and Palade, 1963). Characterisation of molecular structure has led to the identification of different types of endothelial tight junctions and association proteins, namely occludin (Ando-Akatsuka et al., 1996; Furuse et al., 1993; Hirase et al., 1997), claudins (Liebner et al., 2000a; Liebner et al., 2000b; Lippoldt et al., 2000; Morita et al., 1999) and submembranous zona occludens (Fig 1.4) (Balda and Anderson, 1993; Haskins et al., 1998; Jesaitis and Goodenough, 1994; Stevenson et al., 1986). Both 7H6 (Zhong et al., 1994) and cingulin (Citi et al., 1989) are also tight junction association proteins, firstly characterised in the periphery and have also been observed to be located within the endothelial BBB (Wolburg and Lippoldt, 2002).

Occludin was the first tight junction molecule to be discovered, although its role is not yet fully characterised (Furuse et al., 1993). Studies have provided

evidence that structural formation of occludin is not necessary for the formation of the BBB but maintains a role in regulation of BBB properties (Lacaz-Vieira et al., 1999; Saitou et al., 2000).

The claudins are a family of tight junction molecules and are associated with the formation of the endothelial BBB and its barrier properties in many studies (Furuse et al., 1998a; Furuse et al., 2001; Furuse et al., 1998b; Furuse et al., 1999; Morita et al., 1999; Tsukita and Furuse, 1999). Claudin -1 and -5 have been located within endothelial cells of the brain (Liebner et al., 2000a; Liebner et al., 2000b; Lippoldt et al., 2000; Morita et al., 1999), as well as peripherally. Structurally these proteins share 4 transmembrane domains with occludin, but they do not contain sequence homology (Wolburg and Lippoldt, 2002).

Zona occludens (ZO) are tight junction association proteins, as they tether tight junctions to the cytoskeleton (Furuse et al., 1994; Itoh et al., 1999). These proteins are classified to form 3 members- ZO-1, -2 and -3 and are membrane associated guanylate kinases (MAGUK) (Wolburg and Lippoldt, 2002). There have also been reports that ZO interacts with adhesion molecules such as junctional adhesion molecule (JAM) -1 (Bazzoni et al., 2000; Hamazaki et al., 2002). Adhesion molecules include JAM, platelet endothelial cell adhesion molecule (PECAM) -1 and endothelial cell-selective adhesion molecule (ESAM), which are also localised at tight junctions (Hirata et al., 2001; Martin-Padura et al., 1998).

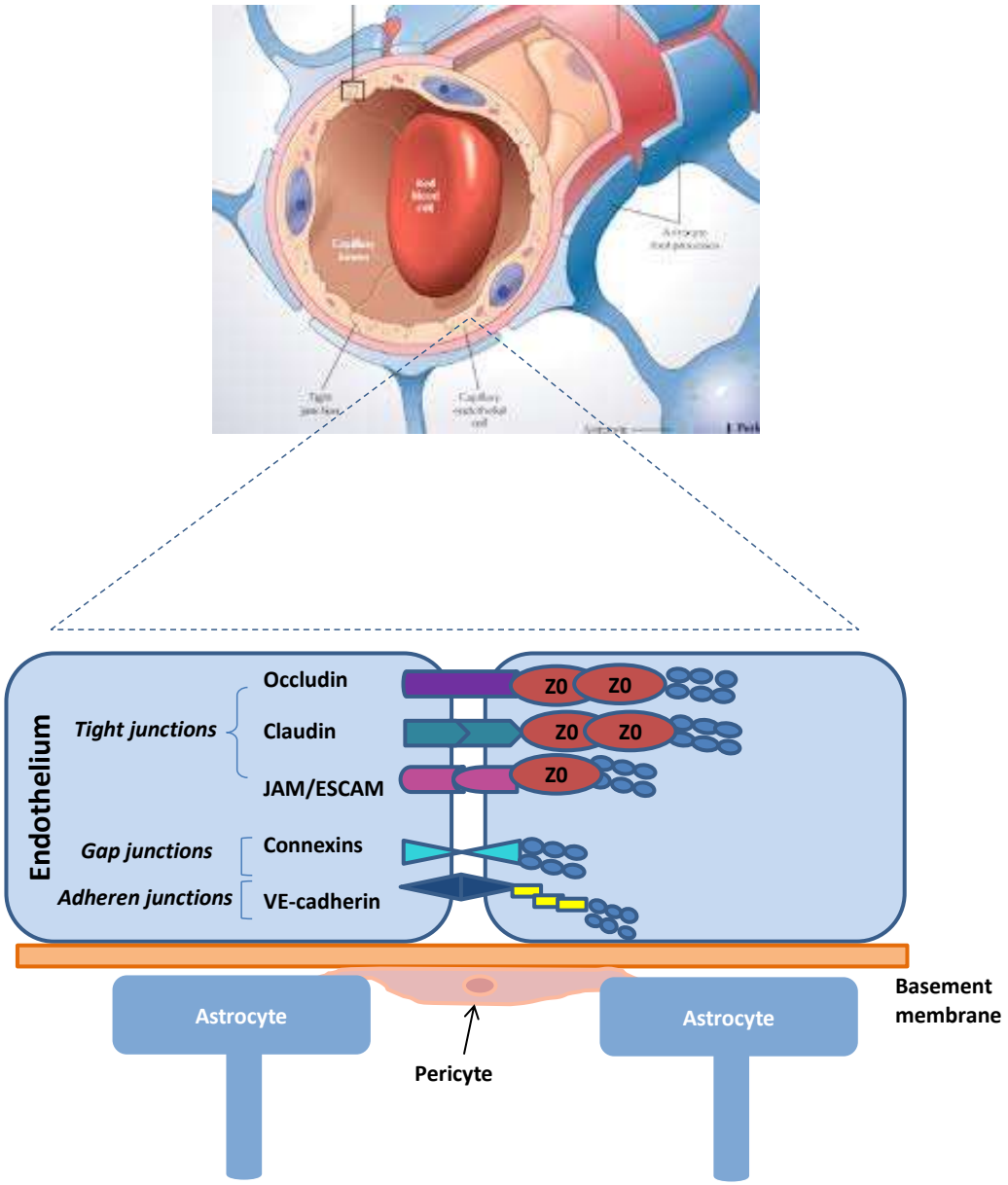

Figure 1.4: The endothelial blood-brain barrier

Schematic of the endothelial BBB. One key feature of the cerebrovasculature is the endothelial blood-brain barrier, which prevents the entry of toxic and harmful products into the brain. The endothelial cells are organised in a line within the blood vessels and are connected by tight junctions, gap junctions and adheren junctions, forming the properties of the BBB. (Image adapted from www.studyblue.com)

1.3.3. Glia

The other type of cells which form part of the neurovascular unit are the glia, the most numerous group of non-neuronal cells within the brain, forming active partners with neurons, providing multiple roles such as maintaining environmental homeostasis, cell-cell signalling, trophic support, innate immunity, regulation of blood flow and ensuring signals sent from neurons evoke the correct vascular response (Bezzi and Volterra, 2001).

1.3.3.1. Astrocytes

Astrocytes are uniquely positioned as an anchor between neurons and blood vessels. Astrocytes form specialised end-feet, which cover around 90% of the vascular abluminal membrane, contacting both neurons and synapse (Kacem et al., 1998; Peters et al., 1991). On blood vessels, these astrocytic end-feet become anchored to components of the basal lamina and play a crucial role in volume and ion regulation (Abbott et al., 2006). One mechanism by which astrocytes can induce alterations to blood flow is by their response to glutamate released by neural activity, activating astrocytic metabotrophic glutamate receptors, leading to increased Ca^{2+} and local vasodilation (Abbott et al., 2006; Takano et al., 2006; Zonta et al., 2003). Astrocytes also use Ca^{2+} signalling to activate other surrounding glia such as oligodendrocytes and microglia (Hansson and Ronnback, 2003).

1.3.3.2. Microglia

In the normal brain, microglia are located sporadically, surrounding arterioles and capillaries, forming one of the cellular components of white matter (Esiri and McGee, 1986). Microglia are the main form of innate immune response and are extremely sensitive to alterations within their local microenvironment.

Morphologically, under normal conditions microglia have a small circular cell body, in conjunction with outwards extending processes (Garden and Moller, 2006; Perry and Gordon, 1988). These cells were previously thought to be in a 'resting' state, but through advances in in-vivo imaging, these cells have been shown to be continually 'surveying' their microenvironment by extending and contracting their processes (Davalos et al., 2005; Nimmerjahn et al., 2005). In response to alterations in the homeostasis of their microenvironment, which may be due to CNS injury, ischemia, inflammatory stimuli or cellular signalling, microglia are recruited and differentiate into an active phenotype, migrating to the source of injury. Once recruited to the site of injury, microglial cells are able to phagocytose dead or dying cells and their debris, which can lead to the enlargement of their cell bodies. Recruitment of microglia can also be self-perpetuating, in that active microglia release a range of inflammatory mediators, which further recruit microglia (Garden and Moller, 2006).

1.3.3.3. Oligodendrocytes and myelinated axons

Oligodendrocytes play a critical role in the connectivity of the CNS by producing the myelin sheath. Myelin is a substance composed of a high lipid content (70/30%, lipid to protein ratio) and low water content. The composition of myelin is the reason for the characteristic 'pale white' colour of WM and a feature that multiple imaging techniques such as magnetisation transfer imaging (MRI) have taken advantage of (Barkhof and Scheltens, 2002; Quarles et al., 2006).

In the normal brain, the oligodendrocytes form the myelin sheath by extending their myelin comprising processes onto neighbouring axons. Once in contact with the axon, the processes of the oligodendrocyte 'ensheaths' and 'wraps'

around the axons to form a multilayer insulting sheath. This insulting sheath allows for rapid propagation of axon potentials, which are 100x faster in comparison to non-myelinated axons, allowing faster signal transfer between distant regions of the brain (Baumann and Pham-Dinh, 2001; Fields, 2008; Hodgkin, 1951) and shelters axons, preventing them from exposure to toxic stimuli (Nave, 2010). The myelin sheath does not cover the entire length of the axon, periodic 'gaps' exist termed the Nodes of Ranvier, with the segments of myelin in between known as the internodes. Although species and location dependent, these internodes are round 100-1700 μm in length compared to the Nodes of Ranvier, which are within the region of 1μm and these gaps function to facilitate saltatory conduction of action potentials along the axon, as leaps of current from one Node of Ranvier to the other (Edgar and Nave, 2009).

The abundance of oligodendrocytes within the CNS does not limit them to the myelination of a single axon. In fact, they can extend their processes to multiple axons and likewise, each axon can be myelinated by multiple oligodendrocytes (Peters, 1991). The calibre of the axon also affects the type of oligodendrocyte producing the myelin, with oligodendrocytes categorised into type I to IV based on the size of axons they myelinate, ranging from small to large diameter axons respectively (Butt et al., 1995).

Additionally, oligodendrocytes also provide trophic support to neurons (Du and Dreyfus, 2002) and link neurons to capillaries through connections with astrocytes (Paspalas and Papadopoulos, 1998). Furthermore, oligodendrocytes are able to use Ca^{2+} signalling to signal to surrounding glia (Verkhratsky and Kettenmann, 1996) and have been suggested to release multiple growth factors, such

as brain derived neurotrophic factor and insulin like growth hormone (Hansson and Ronnback, 2003).

In the normal brain myelin develops as we age. At birth, the human brain consists of a small amount of myelin with development of myelin in a heterochonological pattern, until the 5th decade of life. The temporal and parietal lobes are the last to myelinate and have shown to be the first to be lost with age (Marner et al., 2003). This loss of WM integrity, commonly referred to as WML, with age is strongly associated with decreased cognitive function in many long term epidemiological studies, such as The Rotterdam scan study, Honolulu Asia aging and Lothian birth cohort 1936 (De Groot et al., 2000; Deary et al., 2007; Launer et al., 1995). These studies have utilised imaging techniques and found that WML occur with ageing at a frequency of 50% in individuals over the age of 65 (Enzinger et al., 2006). Although the cause of WM loss with age is unknown, imaging studies have investigated risk factors, which may predispose individuals to WM loss and cognitive decline. In particular, vascular risk factors have been associated with the formation of WML, with a large body of evidence pointing towards hypertension being a major risk factor (Pantoni and Garcia, 1995).

1.4. The structural integrity of the hypertensive brain

Hypertension has been associated with causing deleterious effects to the structural integrity of the brain, being the number one risk factor for stroke and age related cognitive decline (Dahlof, 2007). Recent advances in our knowledge of neurovascular regulation and the concept of the neurovascular unit has led to clues of how hypertension may lead to disruption of the structural and functional integrity of

the brain. It has been proposed that hypertension leads to deleterious alterations to the cerebrovasculature, associated with the promotion of atherosclerotic plaques in the cerebral arteries and arterioles, which may progress to arterial occlusions and ischemic injury (Dahlof, 2007; Lammie, 2002). In turn, hypertension has also been associated with inducing fibrinoid necrosis (lipohyalinosis) of the penetrating arteries and arterioles, the main vascular supply to the WM leading to small WM lesions and depending on the severity, brain haemorrhage. These observations are largely credited to Miller Fisher who characterised hypertension related vascular alterations, by the examination of post-mortem brains from hypertensive patients (Fisher, 1969, 1972, 1977; Fisher, 1978; Fisher, 1982; Fisher, 1998; Fisher and Caplan, 1971; Fisher and Tapia, 1987). Fisher examined the vasculature by serial section reconstruction of the blood supply and observed that lacunar infarcts are located distal to occluded small perforating arteries. Fisher characterised two main types of vascular lesions namely: intracranial atherosclerosis and lipohyalinosis, which were associated with lacunes. Future studies described these alterations to the cerebrovasculature as vasculature remodelling, characterised as the ability of the vessel wall to re-organise both cellular and extracellular components in response to a chronic stimulus (Gibbons and Dzau, 1994).

Firstly, Fisher identified that hypertension appears to drive atherosclerosis into the most distal cerebrovasculature observing large lacunes associated and thus attributed to atherosclerotic plaques in 200 to 800μm diameter vessels. Additionally, Fisher identified another destructive lesion of segmental disorganisation found in smaller perforating vessels (40-300μm in diameter) named lipohylinosis, which were

commonly located in the striatocapsule and thalamus being attributed to over two thirds of lacunes identified (Fig 1.6) (Fisher, 1969; Fisher, 1978).

Many of these lipohyalinotic lesions observed were old and characteristic by the loss of normal wall architecture, collagenous sclerosis, mural foam cell accumulations and microbleeds. These features may represent early evidence of alterations to the BBB and it was also postulated that these lesions may lead to alterations in blood flow. These lipohyalinotic lesions were traditionally viewed as a consequence of severe hypertension, but have been found in relation to lacunar infarcts, even in the modern era of 'controlled' treated hypertension (Fisher, 1991; Lammie et al., 1997; Masuda et al., 1983). Pathological evidence has suggested that there is an acute non-healed form of lipohylinotic lesions known as fibrinoid necrosis, which has been found to be most commonly associated with hypertension (Feigin I, 1959; Fisher, 1971; Masuda et al., 1983; Rosenblum, 1977; Takebayashi and Kaneko, 1983). Identification of fibrinoid necrosis requires the use of histological techniques and has been characterised to be circumferential, focal or segmental and particularly prevalent in the basal ganglia; as a result of moderate hypertension and within the pons; as a result of severe hypertension (Feigin I, 1959; Masuda et al., 1983; Rosenblum, 1977).

Throughout the literature the term lipohyalinosis has been wrongly used for a variety of small vessel pathologies. Mostly, the common small vessel pathology characterised by concentric hyaline wall thickening of the small arteries and arterioles, which is most commonly found in the aged brain and has been associated to be exacerbated by hypertension, diabetes and other vascular disorders (Fig 1.6) (Lammie, 2002). The concentric wall thickening known as hyaline arteriosclerosis

begins in the forth decade of life and has been shown to increase in severity with age (Baker and Iannone, 1959a, b; Furuta et al., 1991).

Clinically these small vessel lesions are defined in severity by wall thickness to lumen ratio, which has been found to correlate with systolic blood pressure. Pathological investigations have found these vasculopathies to consist of: degeneration of tunica media smooth muscle cells and internal elastic lamina degeneration, which are replaced by fibroblasts, deposits of collagens I, III, V, VI and basement membrane specific; lamina and collagen IV (Zhang and Olsson, 1997). In the brains of hypertensive individuals, these vascular alterations may be preceded by 'onion skinned' concentric smooth muscle cell proliferation (Kalimo et al., 1997). Overall, the vasculature is left elongated, tortuous, stiffened and remodelled with decreased external wall diameter.

In the modern day, hypertension is still associated with these cerebrovascular alterations, however, to date small vessels pathologies have been studied post mortem, preventing the identification of the initial mechanisms, rather the gross structural alterations, which are readily identified by neuroimaging and are taken as markers of hypertensive related vascular pathology (Pantoni, 2010).

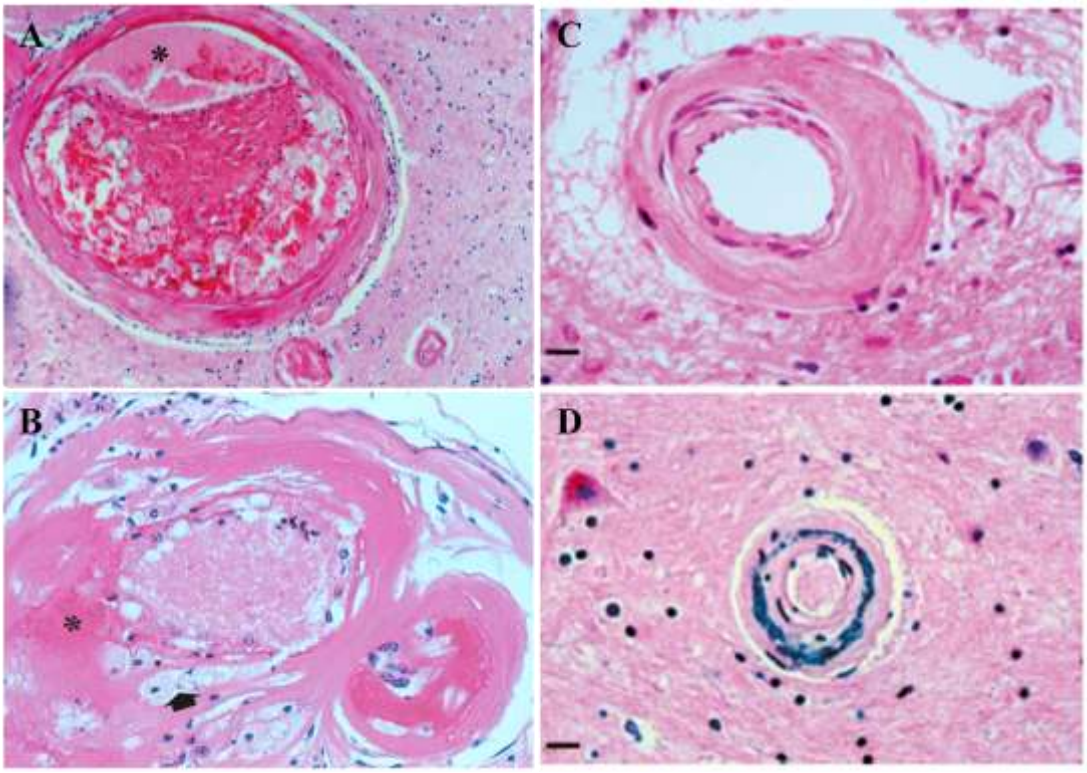

Figure 1.5: Small vessel pathologies in the human brain

Histological evidence of small vessel pathologies. (A) Small vessel atherosclerosis in the putmen -an eccentric atherosclerotic plaque within a penetrating vessel. The presence of the atherosclerotic plaque leads to luminal narrowing (*) and occlusion of the vessel. (B) Lipohyalinosis in the basal ganglia - vessel wall appears thickened and dilated, with overall general disorganisation. There is also focal fibrosis, fibrinoid degeneration (*) and commonly mural cell infiltration (arrow). (C-D) Images within the globus pallidus- arteriolosclerosis, (C) concentric hyaline wall thickening, loss of layers of smooth muscle cells and often moderate luminal narrowing, (D) characteristic of an aged brain, with mineralisation of the tunica media and luminal narrowing. (Image taken from Lammie., 2002)

1.4.1. Vascular inflammation

In addition with hypertension being associated with alterations to the structural integrity of the cerebrovasculature there is also a large body of evidence linking hypertension and vascular inflammation. Although it is not fully understood why there is a link between hypertension and vascular inflammation it may represent a response of the brain to vascular alterations. Clinically hypertensive patients have been found to have increased serum levels of inflammatory markers such as C-reactive protein, which were found to correlate to increased blood pressure (Bautista et al., 2001), (Boos and Lip, 2006; Li, 2006; Sung et al., 2003). Although limited, lesion characterisation studies in hypertensive brains have found that the vessel wall becomes a compressed cavity with a gliotic, neovascularised, fibrotic peripheral zone (Takasugi et al., 1985). In addition, Akiguchi et al., carried out pathological assessments of the structural integrity of the brain from patients who had suffered cerebrovasculature disease (Akiguchi et al., 1997). Their observations were in agreement with early characterisations carried out by Brun and Englund, finding numerous activated microglia and macrophages associated with the cerebrovasculature in areas with signs of WM loss (Brun and Englund, 1986).

Interestingly, the arteriosclerotic lesions originally characterised by Fisher, were described to show endothelial proliferation, splitting of the lamina densa with vascular leakage and marked accumulation of lymphocytes and macrophages in the surrounding area (Fisher, 1969; Fisher, 1978; Fisher, 1991). From the description it could be hypothesised that the leakage of plasma from the vasculature was due to BBB disruption, leading to a recruitment of inflammatory cells.

As described previously, the endothelium plays many crucial roles within the CNS, influencing vascular tone and inflammatory response through signalling mechanisms. Oxidative stress is also associated with endothelial dysfunction and vascular remodelling in hypertension (Cai and Harrison, 2000; Ross, 1999). During vascular remodelling (in particular atherosclerosis), endothelial dysfunction is an initial event followed by vasoconstriction, leukocyte and platelet aggregation (Bonetti et al., 2003; Gonzalez and Selwyn, 2003; Heitzer et al., 2001). The main associated cause of endothelial dysfunction is decreased bioavailability of NO, a potent vasodilator that inhibits leukocytes and platelet adhesion and can release modulatory signalling, controlling smooth muscle cell proliferation (Heitzer et al., 2001). Alterations in NO found in cerebrovascular disease may be due to increased breakdown of NO by reactive oxygen species (ROS), forming the highly reactive toxic peroxynitrite. The production of ROS can also contribute to the uncoupling of the nitric oxide synthase enzymes, crucial for the formation of NO, leading to a further increase in levels of ROS (Munzel et al., 1997). In turn, increased levels of ROS can lead to cellular damage and have highly negative consequences to the structural integrity of the vasculature (Portaluppi et al., 2004).

This is highly relative to hypertension as patients are consistently found to express higher circulating levels of plasma ROS, such as malondialdehyde, 8-isoprostane and 8-oxo7,8-dihydro-2-deoxyguanosine, and these markers have been associated with the severity of vascular alterations (Portaluppi et al., 2004; Redon et al., 2003). In turn, hypertension has been found to be associated with the activation of members of the redox signalling pathways such as mitogen-activated protein (MAP) kinase and the extracellular-signalling related kinase ½ (ERK) pathways,

which influence the vascular expression of NO through the expression of endothelial specific nitric oxide synthase (eNOS) (Lassegue et al., 2001; Ungvari et al., 2003).

Increased levels of ROS associated with hypertension can stimulate an inflammatory response through the expression of transcription factors such as NFκB, an inflammatory mediator that can stimulate the expression of inflammatory cells. In turn, inflammatory factors such as IL-6 and IL1β can induce endothelial dysfunction (Bhagat and Vallance, 1997; Cleland et al., 2000; Hurlimann et al., 2002; Paffen and deMaat, 2006; Wang et al., 1994). There is at present an overwhelming association with hypertension, vascular remodelling, oxidative stress and vascular inflammatory. However, the exact mechanisms as yet are not fully elucidated and it would be interesting to carry out studies investigating alterations to vascular related genes before and after signs of vascular remodelling.

1.4.2. Hypertension related vascular alterations and associated gene changes

With recent advances in gene profiling techniques the ability to investigate alterations in gene expression with hypertension and in comparison to structural alterations, has provided a new approach to identify the underlying mechanisms. As described previously, the initial genetic studies identified a few candidate genes associated with hypertension, which are related to the renin-angiotensin-aldosterone system (RAAS), kallikrein-kinin system and ion transport (Adragna and Lauf, 2007; Ji et al., 2008; Schild et al., 1996), with many of these gene alterations forming the basis of pharmacological targets (Arnett and Claas, 2009; Levy et al., 2009).

Many large Genome wide association studies have been carried out to investigate alterations in gene expression in individuals with hypertension but have

been relatively unsuccessful, due to the inherent variation in population samples according to duration of hypertension, age and vascular pathology. The CHARGE Consortium GWAS included 29,136 participants aged between 38-72, whom had previously undergone blood pressure measurements in 6 population based studies: Age, gene/Environment Susceptibility Reykjavik study (AGES), Atherosclerosis Risk in the Communities (AIC) study, Cardiovascular Health study (CHS), Framingham Heart study (FHS), Rotterdam Study (RS) and the Rotterdam Extension Study (RES) (Levy et al., 2009). In general, these studies provided evidence of alterations to the RAAS, Ion transport, Calcium and NO signalling pathways with hypertension (Levy et al., 2009; Salvi et al., 2012). Furthermore studies have also identified missense variants of the NO endothelial receptor, eNOS in hypertensive patients (Lacolley et al., 1998; Shimasaki et al., 1998; Yoshimura et al., 1998) and more specifically the HYPERGENES European population study, in which hypertensive patients were followed for a period of 5-10 years, found several eNOS related single nucleotide polymorphisms, and in particular the rs3918226, were strongly associated with hypertension. This study also found a significant interaction between the expression of rs3918226 and the expression of both actin and heat shock protein genes in hypertensive patients (Salvi et al., 2012). Overall the current studies have identified genes, which are altered with hypertension but it is unknown if these genes are associated with causing hypertension or caused by hypertension.

1.4.3. White matter integrity and hypertension

Not only is hypertension thought to alter the structural integrity of the cerebrovasculature but these alterations are thought to underlie WML, thus making hypertension a risk factor for the formation of WML. Imaging studies frequently

observe WML in elderly brains and these are associated with decreased cognitive function. Additionally, arteriolosclerosis of the small penetrating arteries and arterioles, (which hypertension is thought to target), is commonly found in brains of the elderly with diffuse WML (Pantoni, 2010). Thus, establishing the role of hypertension in the formation of WML is critical.

Our knowledge of the structural alterations to the WM with age and ability to correlate these changes to vascular risk factors has been influenced by advances in in-vivo imaging techniques, such as CT and MRI (Figure 1.7) (Pantoni and Garcia, 1995). The co-application of imaging findings and post-mortem pathological studies has characterised these WML as areas of demyelination, loss of oligodendrocytes and vaculation (Brown and Thore, 2011; Brun and Englund, 1986; Pantoni and Garcia, 1997a).

In general, the use of these imaging techniques has lead to a large body of evidence showing an association between hypertension and the severity of WML. Early studies using CT imaging found that a large number of individuals with WML were hypertensive and also exhibited impaired cognitive function (Blennow et al., 1991; Bogousslavsky et al., 1987; Hijdra et al., 1990; Rezek et al., 1987). With the induction of MRI scans many more studies have been carried out and have shown a strong correlation. Namely the atherosclerosis risk in the communities study, which found that around a third of the population aged 55-72 years had WML, with half of those hypertensive (Liao et al., 1996) and similar findings have reported in both The Cardiovascular Health study (Longstreth et al., 1996) and Rotterdam study (Breteler et al., 1994). As previously stated WML do occur in normal aging and in the absence of hypertension (Breteler et al., 1994; Liao et al., 1996; Longstreth et al., 1996;

Sierra et al., 2002), but at a lower rate and severity (Liao et al., 1996; Pantoni and Garcia, 1995). Therefore, hypertension is thought to exacerbate features of the ageing brain.

Further evidence supporting hypertension as a risk factor for WML was established by a study carried out by Sawn et al., finding that hypertension in midlife, predisposed individuals to WML in late life. They examined a population of 68-79 community dwelling men, carrying out MRI examinations and cognitive testing over a 10 year period. It was found that those subjects who had high midlife systolic blood pressure, experienced a greater severity and prevalence of WML with a corresponding decrease in cognitive function in late-life, reinforcing the hypothesis that hypertension midlife is a predictor for WML in late-life (Swan et al., 1998). Nonetheless, vascular risk factors such as diabetes (which often co-exists with hypertension in humans), have been linked with the presence of WML (van Harten et al., 2006), proposing that several different pathogenic pathways may be present in the formation of WML.

The evidence that hypertension alters CBF further supports the association due to the anatomical location of WML being frequently found within the periventricular and subcortical regions of the brain (De Groot et al., 2002; De Leeuw et al., 2002; Matsushita et al., 1994; van Dijk et al., 2004), which have been found to be sensitive to alterations in CBF (Pantoni and Garcia, 1995). Histological studies have been undertaken to correlate WML observed on CT and MRI scans with autopsy findings and these studies have identified pathological differences between periventricular and subcortical WML (Pantoni and Garcia, 1995). Periventricular WML correlated with decreased myelin, gliosis, axonal loss, increased extracellular

fluid and ventricular enlargement (Chimowitz et al., 1992; Fazekas et al., 1991; Fazekas et al., 1993; Grafton et al., 1991; Jungreis et al., 1988; Leifer et al., 1990; Moody et al., 1995; Scarpelli et al., 1994; Sze et al., 1986; Van Swieten et al., 1991). Deep subcortical white matter abnormalities on the other hand, correlated with enlarged perivascular spaces, small infarcts, cysts, and de-myelinated plaques with no evidence of axonal loss (Braffman et al., 1988; Chimowitz et al., 1992; Jungreis et al., 1988; Marshall et al., 1988; Munoz et al., 1993; Scarpelli et al., 1994) . There was also evidence of astrogliosis and thickening of the wall of small vessels (Fazekas et al., 1993; Leifer et al., 1990; Lotz et al., 1986; Marshall et al., 1988; Revesz et al., 1989; Van Swieten et al., 1991).

In addition further supporting the link between hypertension and WML, many studies have found antihypertensive treatment beneficial in preventing the formation and progression of WML. It has been shown that treated hypertensive patients with controlled blood pressure have a lower volume and decreased severity of WML, when compared to untreated hypertensive patients (Liao et al., 1996). The Perindopril protection against recurrent stroke study (PROGRESS), used Perindropril; a long acting angiotensin converting enzyme (ACE) inhibitor and examined its ability to prevent WML. Patients exposed to this form of anti-hypertensive treatment had a reduction in the formation of new WML compared to untreated hypertensive individuals (Dufouil et al., 2005), although the specific mechanisms are not yet known. Many other studies have examined different anti-hypertensive treatments and have found similar results, suggesting that controlling hypertension is important to help prevent or alleviate structural alterations to the

brain. However, antihypertensive medications have a broad spectrum of targets therefore this effect cannot be exclusively attributed to blood pressure lowering.

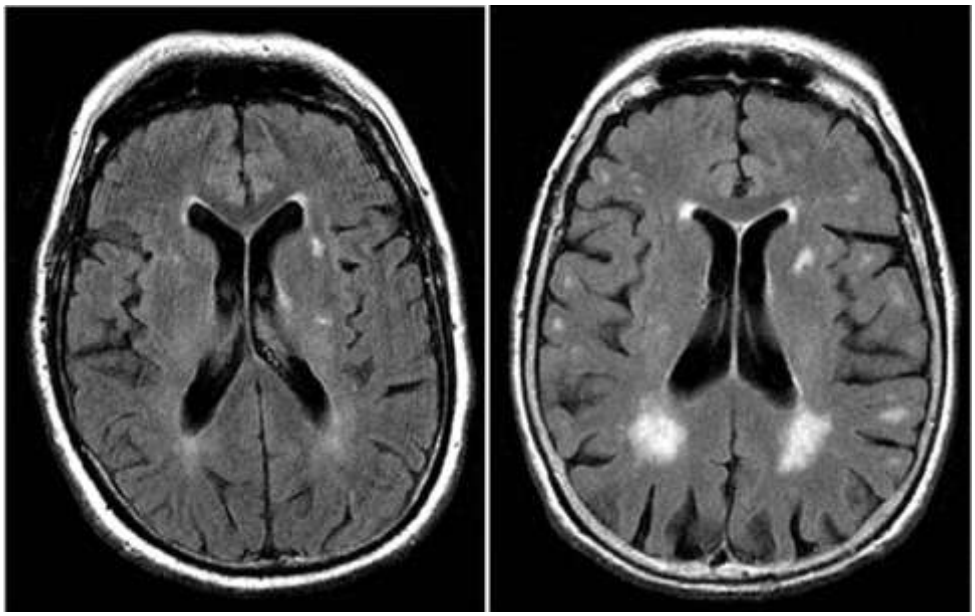

Figure 1.6: White matter lesions
Magnetic resonance imaging (MRI), showing different severities of white matter hyperintensities, in two 80 year old patients. The hyperintensities appear as bright white spots, which reflect areas where tissue contains more fluid than the surrounding tissue, with pools of free water and these spots are thought to represent white matter lesions within white matter tracts of the brain. White matter lesions are frequently found within the periventricular and deep subcortical regions of the brain. White matter lesions can be seen in the above image with the patient on the left having a lower severity, than those observed to be more extensive in the patient on the right. (Image taken from (Debette and Markus, 2010)).

1.4.4. Hypertension and cognitive function

Cognitive decline is becoming an ever important issue in our aging society, with hypertension associated as a risk factor for cognitive decline. In addition to WML, increased blood pressure and decreased cognitive function are two attributes commonly found with increasing age particularly in those ≥60 years of age and beyond (Botwinick, 1977; Kausler, 1982), with hypertension firstly being associated with cognitive decline in individuals over 60 years of age several decades ago (Wilkie and Eisdorfer, 1971).

The most robust evidence has come from longitudinal epidemiological studies, with the majority of studies finding a relationship between midlife hypertension (around 50 years of age) and cognitive decline in late-life (see table 1.1). One of the first longitudinal studies, to find a correlation between initial blood pressure measurements and the development of cognitive decline a decade later was the Framingham Study (Elias et al., 1993). Subsequently, a 20 year long population-based study found that cognitive function at the age of 70 was inversely related to initial arterial blood pressure measurements at 50. Therefore, high blood pressure at midlife was associated with cognitive decline in late-life (Kilander et al., 1998a).

The epidemiology of vascular aging (EVA) population study is another key study examining hypertension in relation to cognitive decline. Using a shorter follow up period of 4 years, they found that patients who had high blood pressure at baseline exhibited cognitive decline after the 4-year follow-up. Interestingly, it was found that hypertensive patients had a 2.8 times higher risk of developing cognitive decline than normotensive individuals (Tzourio et al., 1999). Although, the EVA study examined a small cohort, these results have been confirmed in the far larger Atherosclerosis

risk in communities study (ARIC), with a follow up period of 6 years and found that hypertension and diabetes are associated with the formation of cognitive decline (Knopman et al., 2001).

Overall, the studies that provided evidence of cognitive decline as a result of hypertension showed impairments in attention, learning and memory, executive function, visuospatial skills, psychomotor abilities and perceptual skills (Deshmukh et al., 2009; Elias et al., 2004; Waldstein, 2003). These impairments in attention, perceptual processing and executive function, can be associated with cognitive deficits frequently found in individuals with subcortical vascular dementia, whom exhibit pathological alterations to deep subcortical WM circuits (Cummings, 1998).

Furthermore, within the literature there was a discrepancy as to whether high or low blood pressure is more detrimental to cognitive function. A study by Skoog et al., provided some clarity demonstrating that high blood pressure measurements found in individuals ~70 years of age were associated with a high prevalence of developing cognitive decline, specifically dementia some 10-15 years later, than those whose blood pressure was within a normal range (Skoog et al., 1996). Interestingly this study found that upon the development of dementia symptoms, individuals who had previously been reported as having high blood pressure had developed low blood pressure.

Further supporting evidence of a link between hypertension and cognitive decline has come from the findings that anti-hypertensive treatment can act to preserve cognitive function. Findings from non-randomised trials are overall inconsistent, initially reporting that anti-hypertensive medications lead to an adverse

affect on cognitive function (Khachaturian et al., 2006; Lindsay et al., 2002; Mielke et al., 2007; Morris et al., 2001; Qiu et al., 2003). In general, most of the observational studies, which have been carried out have found that anti-hypertensive medications are neuroprotective to cognitive functions. This has been shown in the EVA cohort study, in which the risk of developing cognitive decline after a 4-year follow-up was significantly decreased in treated versus non-treated hypertensive's (Tzourio et al., 1999) and a 5-year period with treatment is associated with a 38% reduction in cognitive impairment compared to non-treated hypertensive patients (Murray et al., 2002).

Several large placebo-controlled clinical trials have been carried out but overall the results of these trials are heavily dependent on the type of treatment used, duration of hypertension and follow-up period. The first study to find a reduction in development of cognitive decline was the SYST-EUR trial, examining patients >60 years of age with isolated systolic hypertension, receiving treatment with a calcium channel antagonist, combined with an angiotensin converting enzyme inhibitor; enalapril and/or a diuretic; hydrochlorothiaziade, compared to over 1000 placebo treated patients. Over a 2-year period, the incidence of dementia was remarkably decreased by 50% in the treated group compared to the placebo group (Forette et al., 2002; Forette et al., 1998). There have been several other clinical trials which aim to validate and expand on the above finding, such as the HOPE trial (Bosch et al., 2002), PROGRESS (Tzourio et al., 2003) and SCOPE (Lithell et al., 2003), with regard to increasing severity of hypertension and comparisons between different medications. Overall, the majority of data indicates that hypertension can impact negatively on cognitive abilities.

Key longitudinal studies investigating the association between hypertension and cognitive decline

Reference	Age (years)	Follow-up (years)	Association
Elias et al. 1993	55-88	12-14	Yes
Launer et al. 1995	20	20-28	Yes
Skoog et al.1996	≥70	10-15	Yes- high blood pressure at 70 predicted cognitive decline some 10-15 years later
Starr et al. 1997	>69	4	Yes
Guo et al. 1997	75-101	3	Yes- J curve relationship
Kilander et al. 1998	50	20	Yes
Swan et al. 1998	45	25-30	Yes
Tzourio et al. 1999	59-71	4	Yes
Glynn et al. 1999	65-102	9	Yes- J curve relationship
Knopman et al. 2001	47-70	6	Yes
Bohannon et al. 2002	65-105	3	Yes- J curve relationship
Reinprecht et al. 2003	68	13	Yes
Piguet et al. 2003	≥75	6	Yes
Elias et al. 2003	55-88	4-6	Yes
Tervo et al. 2004	60-76	3	No
Solfrizzi et al. 2004	65-54	3.5	No
Waldstein et al. 2005	70	6	Yes
Kuo et al. 2005	65-94	2	Yes
Waldstein et al. 2008	57	5	Yes
Goldstein et al. 2013	72-75 (mean age)	2	Yes

Table 1.2: Key longitudinal studies examining cognition in relation to hypertension

The above table shows the findings from several studies examining cognitive decline and dementia.

1.5. Animal models of hypertension

Hypertension in humans commonly occurs concomitantly with other vascular risk factors, such as diabetes, which has also been associated with cerebrovascular pathology. For this reason, animal models are required to unravel the mechanisms of hypertension in 'isolation' on the structural and functional integrity of the brain.

Due to the multifactorial nature of hypertension, many animal models have been created by genetic, pharmacological and surgical manipulation (see (Lerman et al., 2005) for an in-depth review of hypertensive animal models). In general, rat models are most commonly used due to the similarities in the structure of the Circle of Willis in comparison to humans (Figure 1.8). The best characterised experimental models of hypertension are the spontaneously hypertensive rat (SHR) and (SHR) stroke prone (SHRSP), therefore they will be the extensively covered models in this thesis. These models have dramatically improved our knowledge in this field and have remained the gold-standard models for several decades. However, the limitations of these models have left the initial mechanism of hypertension on the structural integrity of the brain unknown. Thus, this thesis sought to investigate the effect of hypertension on the structural and functional integrity of the brain using the Cyp1a1 Ren2 rat model, in which the severity and duration of hypertension can be tightly controlled and compared to strain matched litter-mate controls.

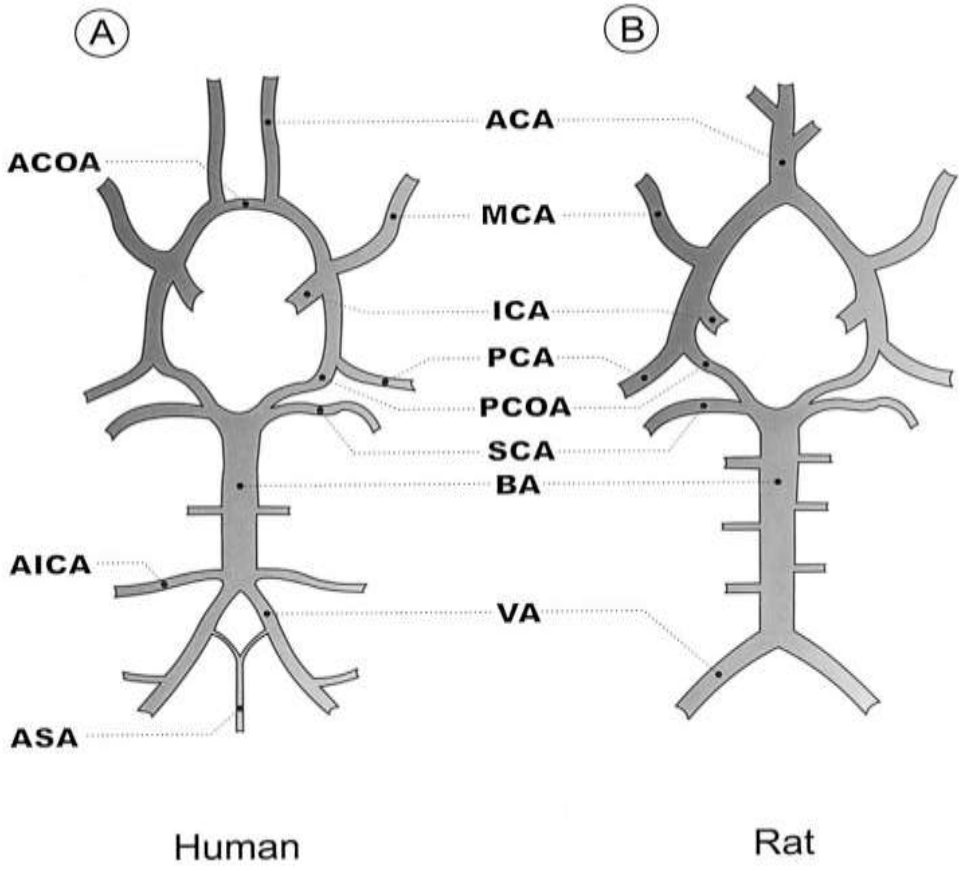

Figure 1.7: The anatomy of the Circle of Willis in humans and rats

The Circle of Willis is relatively similar between humans and rats, allowing rats to be used to study conditions which alter the cerebrovasculature and cerebral blood flow. Some of the differences include the anterior communicating artery, which is found in humans but not rats and the olfactory artery, which is found in rats but not humans. Abbreviations: ACA- anterior cerebral artery, ACOA- anterior communicating artery, AICA- anterior inferior cerebral artery, ASA- anterior spinal artery, BA- basilar artery, ICA- internal carotid artery, MCA- middle cerebral artery, PCA- posterior cerebral artery, PCOA- posterior communicating artery, SCA- superior cerebral artery and VA- vertebral artery. (Image taken from Farkas & Luiten 2000).

1.5.1. The spontaneously hypertensive rat and spontaneously hypertensive stroke prone rat

The spontaneously hypertensive rat (SHR) model was created by the selective breeding of Wistar Kyoto (WKY) rats that were found to have the highest blood pressure compared to other rats. In this model blood pressure rises by the fifth week of life and rapidly reaches levels of systolic blood pressure around 200 mmHg. Although the SHR model exhibits target end organ damage, they are not prone to develop strokes (Okamoto and Aoki, 1963). The spontaneously hypertensive stroke prone rat (SHRSP) is a sub-strain of the SHR bred specifically for the development of malignant hypertension (systolic blood pressure >220mmHg by 8 weeks of age) and stroke (Yamori, 1994; Yamori and Horie, 1977). The phenotype of both the SHR and SHRSP models includes several differentially expressed genes, many of which are unknown, but are known to be associated with the over-reactivity of RAAS (Yamori and Horie, 1977). Overall the SHR and SHRSP models have provided a wealth of knowledge as described below, but there is one main limitation to the majority of these studies in that the control strain used is the WKY, which is not genetically identical and thus strain individual differences exist (Johnson et al., 1995).

1.5.1.2. Animal models of hypertension and cerebral blood flow

In the majority of studies carried out there is evidence of alterations to CBF in both the SHR and SHRSP models. Yamori et al., measured regional cerebral blood flow in SHRSPs using the hydrogen clearance method and found that frontal cortical blood flow was decreased in 60 week old SHRSPs, in those with blood pressure over

200mmHg. This was postulated to be due to alterations in cerebrovascular reactivity, which was found to be decreased in 9 month old SHRSPs (Yamori and Horie, 1977). Similar findings were shown in the SHR model by Fujishima et al., who used the hydrogen clearance technique to measure rCBF in young (3-4 month old) and aged (16-17 month old) SHR. Fujishima et al., found that cortical and thalamic rCBF was decreased in aged SHR, when compared to WKY. This decreased rCBF was asso ciated with structural alterations to the cerebrovasculature and also a deficit in cognitive function (Fujishima et al., 1995a).

In turn, cerebral autoregulation has been found to be altered in the SHR model (Harper and Bohlen, 1984), when compared to normotensive WKY controls. This shift in autoregulation means that with chronic hypertension there is an exacerbation of the vasoconstrictive response to acute increased pressure, which may be an underlying factor of vascular dysfunction (Faraci et al., 1990). The change in cerebral autoregulation has been linked with alterations in the distensibility of the vasculature. With chronic hypertension, the larger arteries of the SHR and SHRSP become stiffened and less distensible, whereas the smaller arterioles become more distensible (Baumbach and Heistad, 1988; Baumbach and Hajdu, 1993; Baumbach and Heistad, 1989). This change in distensibility of the vasculature is linked with structural alterations, which may predispose the brain to a decrease in activity-dependent energy.

Therefore, alterations in CBF may be due to vascular remodelling or altered reactivity. Alterations in cerebrovascular reactivity have been found in the SHR model, which during periods of hypercapnia has an attenuated dilatory response, failing to regulate CBF (Johansson and Nilsson, 1979) and similar findings have

been reported in the SHRSP as a result of increased mean arterial pressure (Sadoshima et al., 1983). Overall there is evidence that CBF is altered in the SHR and SHRSP models, which may be a response of cerebrovascular alterations.

1.5.2. Animal models of hypertension and alterations to the cerebrovasculature

In general, both the SHR and SHRSP models have found cerebrovascular alterations similar to those found in humans with evidence of vascular wall thickening, smooth muscle hypertrophy and BBB disruption. These features were originally observed in initial characterisation studies demonstrating gross cerebrovascular structural alterations in both SHRs and SHRSPs, when compared to normotensive WKY (Okamoto and Aoki, 1963; Okamoto, 1974; Yamori et al., 1976a; Yamori et al., 1976b). Since the development of both the SHR and SHRSP models several studies have been carried out examining how hypertension affects the different populations of vasculature within the brain over different durations. The key findings from these studies will be described below.

1.5.2.1. Animal models of hypertension and large artery structure (≥150μm)

Many of the initial studies focused on examining large arterial structure in response to hypertension, with the most consistent finding being that both the SHR and SHRSP exhibit vascular wall thickening. One of the initial studies was carried out by Nordborg & Johansson, aimed to determine arterial structure both during the development of hypertension, and as a result of hypertension, examining SHRs and WKYs, 15 to 200 days old. Measurements of the vessel wall (media/radius ratio) were calculated from postfixed vessels stained using a modified elastine- van Gieson method. They found that by 15 days of age the large arteries of the SHR model displays increased thickness of the media/radius ratio when compared to age

matched, normotensive WKY (Nordborg and Johansson, 1980), indicating that the vasculature of the SHRs is altered prior to the development of hypertension. These early signs of vascular remodelling have also been reported in the large arteries of SHRSP model at 15 days of age (Okamoto, 1974). Additionally, the external diameter of the carotid artery has also been found to be decreased in SHRSPs, 4-8 weeks of age (Zanchi et al., 1997).

Moreover, vascular wall thickening has also been observed in SHRs and SHRSPs with established hypertension. Nordborg et al., 1985 examined the extraparenchymal cerebral arteries in 7 and 12 month SHRs, SHRSPs and WKYs and found that the vasculature had an increased media/radius ratio, which represented a thicker media and/or smaller internal radius in the SHRs but, only a smaller internal radius in the SHRSPs. In addition Hart et al., carried out wall/lumen ratio measurements in large arteries and arterioles of 13 month old SHRSPs and found evidence of severe vessel hypertrophy, which was most pronounced in the parenchymal vessels (Hart et al., 1980).

Along with the morphological alterations reported above, histological studies have been carried out in an attempt to analyse the corresponding cellular alterations. Mangiarua et al., investigated the cerebral arteries of 28 week old SHRs, SHRSPs and WKYs, using electron microscopy and observed that the hypertensive models exhibited increased number of smooth muscle layers in the basilar and superior cerebral arteries (Mangiarua and Lee, 1992). A following study by Sabbatini et al., analysed the structural integrity of intracerebral arteries in 24 week old SHRs and observed that vessel wall thickening corresponded with hypertrophy of the smooth muscle layer with no change in the number of smooth muscle cells as compared to

normotensive controls (Sabbatini et al., 2001). Additionally immunohistochemical studies have found that smooth muscle actin levels were unchanged in 20 week old SHRs but were markedly increased in the more severe malignant hypertensive SHRSP model (Lin et al., 2001). Overall the above findings in both the SHR and SHRSP models represent that the large arteries undergo structural alterations, although the early occurrence of this prior to hypertension in both models leaves uncertainty as to the impact of hypertension.

1.5.2.2. Animal models of hypertension and the structure of small arteries, arterioles and capillaries (<80µm)

Early lesion characterisation studies in the SHRSP model, observed that cerebrovascular lesions frequently occurred within arterial 'boundary zones' supplied by branching penetrating arterioles and capillaries, commonly located within the subcortical basal ganglia region of the brain, implying that the smaller calibre vessels supplying these regions are susceptible to structural alterations (Yamori et al., 1976b). Evidence of alterations to smaller arterial vessels (≤80 µm) has also been observed by Nordborg & Johansson., examining 200 day old SHR and providing evidence of vascular wall thickening (Nordborg and Johansson, 1980). These vascular alterations have also been found to increase in severity with age as shown by Knox et al., who examined the ultrastructure of the frontal cortical cerebrovasculature in young and aged SHR and WKY. Subtle structural alterations were found within the vasculature of the SHR model aged >3 months, which increased in intensity, frequency and severity with age compared to the WKY. The vessels of SHR were observed to have overt structural alterations, consisting of vascular wall thickening, attributed to collagen deposition and fragmented smooth

muscle cells. Interestingly in agreement with the idea that hypertension exacerbates features of normal aging in humans this study found that cerebrovascular alterations such as wall thickening, were also observed in aged WKY when compared to young, but were reported to be less severe than the hypertensive animals (Knox et al., 1980). These histological findings have also been shown in-vivo by Harper et al., who analysed both structure and functional characteristics of parietal cortical arterioles and capillaries of 18-21 week old SHRs. As with the other studies they found evidence of vascular wall thickening but the main findings of this study were that under normal pressure values the vasculature appeared constricted with no overall change in diameter, whereas during induced decreased pressure the overall diameter of the vasculature was decrease (Harper and Bohlen, 1984).

More recently, a histological study was carried out to investigate cerebrovascular structure in different regions of the brain. Sabbatini et al., examined the cerebrovascular structure in the cortex, striatum and hippocampus of 24 week old SHRs and WKYs, finding regional differences in cerebrovascular alterations. Within the frontal cortex, the vasculature was found to exhibit wall hypertrophy and narrowing of the lumen. On the other hand, the vasculature within the striatum was found to have an overall increased wall area, indicative of hypertrophy without vasoconstriction. Lastly, the hippocampal vasculature was found to undergo vascular remodelling in the form of luminal narrowing (Sabbatini et al., 2001). The regional differences in cerebrovascular remodelling found in this study represent that hypertension in the SHR model does not affect the brain uniformly.

In general, similar vascular alterations have been reported in the SHRSPs as have been found in the SHRs, only more severe. However, in the SHRSP model,

shortly after the onset of hypertension (aged <10 weeks), there are structural alterations to the smaller arterioles (~30μm, in diameter), arising from the lenticulostriate arteries (Mies et al., 1999). In turn, during the development of hypertension, the arterioles have been reported to be decreased in diameter. Coyle et al., injected latex into the MCA of 10 week old SHRSPs to measure the internal diameter of the collateral capillaries and found a marked decrease in the internal diameter of capillaries from the SHRSPs in comparison to WKYs (Zanchi et al., 1997). However, it is unclear if these early vascular alterations are due to the development of hypertension or cause a predisposition for vascular alterations.

To profile cerebrovascular alterations during the development of hypertension, Tagami et al., carried out a characterisation of the penetrating arterioles (<100μm) of SHRSPs aged 4-52 weeks, compared to age matched WKYs. Along with a decrease in the internal diameter of the penetrating arterioles, it was reported that SHRSPs with established hypertension at 16 weeks have focal areas of collagen deposition and cytoplasmic necrosis in the outermost medial layers (Tagami et al., 1987). In turn, the vessels of SHRSPs have also be found to have increased expression of smooth muscle actin at 16 weeks (Bailey et al., 2011). These vascular alterations increased in severity with age, with widespread necrosis and medial atrophy reported at around 28 weeks of age (also shown in (Yamori et al., 1976b)). The outermost medial layers were found to be replaced by basement membrane like material, fibrous collagen and cellular debris. In turn, by 28 weeks the arterioles, in the surrounding areas to cerebral lesions, have been reported to show hyaline degenerations, fibrinoid necrosis and areas of thrombosis (Tagami et al., 1981). Overall, the majority of studies indicate that SHRSPs have decreased vascular

diameter during the development of hypertension and with chronic sustained hypertension.

However, the level of hypertension evoked in the SHRSP model is more severe than the SHR model and ultimately leads to stroke. Tagami et al., also studied the structure of the cerebral perforating arteries in SHRSPs exhibiting signs of stroke, (aged between 28-50 weeks) and compared them to aged matched WKY. The vasculature was found to be severely thickened, narrowed and often occluded. They also described layers of smooth muscle to be lost within the medial layers, which had been replaced by deposition of basement membrane and collegenous-like material. In turn, plasma leakage was observed with surrounding monocyte adhesion and it was reported that this luminal narrowing in 9 month old SHRSPs was accompanied with thrombosis of the deep penetrating arterioles (Fredriksson et al., 1985; Tagami et al., 1987). However, a recent paper examining vascular integrity in stroke free 10 month old SHRSPs found no evidence of vasculopathies in the deep penetrating arterioles, therefore many of the previous vascular alterations may be in relation to stroke (Brittain et al., 2012). Overall evidence exists that both the SHR and SHRSP demonstrate similar features to those observed in the human studies and highlights that the severity and duration of hypertension are critical features.

1.5.2.3. Animal models and the integrity of the blood brain barrier

Along with morphological alterations, cerebral endothelial function has been found to be altered, manifesting as endothelial BBB dysfunction in both the SHR and SHRSP models. One of the earliest reports of impairments to the BBB in the SHR model was from the histopathological study carried out by Knox et al. Alterations to

the BBB were identified through the administration of intravenous horse radish peroxidase (HRP), providing evidence of BBB disruption in the SHRs. In young 3 month old SHRs, the HRP reaction product was found within vessel walls, endothelial cells, perivascular cells (including the processes of astrocytes) and occasionally within perivascular macrophages, with no evidence in the aged matched WKYs examined. In 12 month old SHRs there was more HRP product found within the basal lamina of arterioles, capillaries and venuels, with perivascular macrophages exhibiting large amounts HRP. The BBB disruption further increased with age in 24 month old SHRs, providing evidence of HRP accumulation within vessel wall segments of most arterioles and venuels, spanning 20-30µm. There was also evidence of HRP reaction in endothelial cells, smooth muscle cells, pericytes and perivascular cells (Knox et al., 1980). Interestingly, the aged WKYs also provided evidence of BBB disruption but this was exacerbated in the hypertensive animals, thus further supporting the hypothesis that hypertension appears to exacerbate cerebrovascular alterations which occur with normal ageing.

More recently Ueno et al., examined vascular permeability and endothelial function within the hippocampus and hypothalamus of 3 month old SHRs and SHRSPs (Ueno et al., 2004a; Ueno et al., 2004b). Using the HRP technique they too reported BBB leakage within both regions, localised within abluminal pits and layers of the basal lamina, in both hypertensive models, which was absent in normotensive controls. This study also investigated endothelial dysfunction, by analysing the endothelial glycocalyx, a proteoglycan surface layer surrounding endothelial cells, which was diminished in the hypertensive models compared to controls (Ueno et al., 2011). Ueno et al., also commented on the increased presence of BBB leakage in the

SHRSP model compared to SHRs and highlighted that these alterations were located exclusively within the arterioles.

Similarly Fredriksson et al., examined the integrity of the BBB using evans blue technique and found evidence of BBB leakage in 4 month old SHRSPs, which progressed in severity with age. It was reported that in SHRSPs aged 5-9 months, BBB leakage was found in several vessels, in around 33% of the cohort compared to none in the WKY controls. Fredrinksson et al, commented that, although BBB leakage was found, it was not observed in every vessel and commonly occurred in animals whose blood pressure had reached values higher than 200mmHg (Fredriksson et al., 1985; Fredriksson et al., 1987; Fredriksson et al., 1988). The age at which BBB leakage became more frequent was in contemporaneous, with reports of high frequency of microinfarcts and stroke like symptoms (Ogata et al., 1982). Therefore the majority of evidence in both the SHR and SHRSP models indicates BBB disruption, although the mechanisms are not fully understood.

1.5.2.4. Animal models and vascular inflammation

As described above, the initial characterisation studies in SHRs and SHRSPs analysed the overt structural alterations to the cerebrovasculature. However, many of these studies observed increased numbers of surrounding macrophages, microglia and other inflammatory cells, similar to human studies. Evidence of this has been provided by Knox et al., who examined SHRs 3-24 months old, observing perivascular macrophages surrounding remodelled vessels. It was reported that the number of macrophages increased in aged hypertensive animals and in those with severe vascular pathology. In turn, this study also found evidence of increased

inflammatory cells in aged normotensive rats, but not to the same extent of hypertensive (Knox et al., 1980). Analogous to SHRs, an earlier study by Hazama et al., found proliferation of macrophages and microglia surrounding cerebrovascular lesions in SHRSPs at various ages from around 8 to 52 weeks. Along with severity and duration of hypertension, they also observed an increased proliferation of inflammatory cells in response to areas of BBB leakage (Hazama et al., 1975). These findings have been added to, with immunohistochemical findings, by Lin et al., directly comparing the presence of microglia between SHR and SHRSP at 12 and 20 weeks of age (Lin et al., 2001). Vascular alterations were reported to be minimal in the SHR model, with a small number of microglia associated with the vasculature. On the other hand, the SHRSP were observed to have more severe vascular remodelling and a high prevalence of microglial cells surrounding the vasculature. This form of vascular inflammation associated with vascular remodelling has been found in 16 week old SHRSPs, with concurrent alterations to the endothelial BBB and increased severity in those fed a high salt diet (Bailey et al., 2011). Overall, there is strong evidence that cerebrovascular alterations in the SHR and SHRSP models are associated with an increased inflammatory response.

1.5.2.5. Animal models of hypertension and differential gene expression

Hypertension is a complex polygenetic trait, which can further alter gene expression within the brain. Unmasking the genes which are altered by hypertension in response to structural alterations in the brain would provide novel information about cerebrovascular alterations, and in turn allow for therapeutic insight. Animal models of hypertension can be used to study mechanisms of vascular remodelling by

examining gene alterations. In the last decade, the decreased cost and increased efficacy of microarray technology has driven a number of studies towards gene profiling (Miller and Tang, 2009). Microarrays are miniaturised arrays consisting of thousands of deoxyribonucleic acid (DNA) fragments or signal oligonucleotides, which are attached to a substance and allow for identification of genes within several tissue samples simultaneously. They are complex extensions of the traditional Southern and Northern hybridization blots, and improvements in technology have led to microarrays becoming efficient, cost effective, discriminatory powered, reproducible, sensitive and specific (Miller and Tang, 2009; Schena et al., 1998; Stears et al., 2003).

Due to the novelty of microarray technology, the literature is still within its infancy. However, studies have begun to examine alterations in gene expression during hypertension and in response to vascular alterations. Waki et al., have carried out a serious of microarray examinations of the nucleus tractus solitarius region of the brain in prehypertensive SHRs aged 3 weeks and those with established hypertension aged 15 weeks compared to aged match WKYs. Firstly, they found increased expression of proinflammatory JAM-1 in both prehypertensive and established hypertensive SHRs compared to WKYs. Follow-up studies found increased expression of another inflammatory marker gp39 in SHRs pre-hypertension and in those with established hypertension, but overall there were no differences in the expression of the majority of cytokines and chemokine genes assessed in the pre-hypertensive SHRs compared to WKYs (Waki et al., 2008).

However, the previous studies did not give an insight into gene alterations, which are solely specific to the vasculature in response to hypertension. In an aim to

overcome this, Zhou et al, carried out a microvascular dissection from the whole brain of SHRs and WKYs aged 12 weeks. In general, they found alterations in the expression of genes within the following functional gene categories: metabolism enzymes, kinases, transporter, defence/immunity proteins, receptor binding, signal transducers, enzyme regulators, structural molecules, translation regulators, cell adhesion molecules and stress response. The key genes that were found to be upregulated in the SHR model were various members of the Heat shock protein family, RAAS, TNFα, nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and several cytokines (Zhou et al., 2005; Zhou et al., 2006). As a result of these studies, a novel gene has been identified in the hypothalamus and brain stem of SHR; soluble epoxide hydrolase (sEH), which is thought to play a role in vasodilation (Sellers et al., 2005). Within these series of studies they also compared gene expression in SHR treated with candersartan and found that the main change was a normalisation in inflammatory related gene expression (Ando et al., 2004).

The most recent microarray study examined gene expression within the frontal cortex of prehypertensive 2 month old SHRs and those with established chronic hypertension at 9 months of age. In general, only a small number of genes were differentially expressed between hypertensive and normotensive animals at both ages. Those included genes involved in energy and lipid metabolism, mitochondrial functions, oxidative stress and ischemic response but did not provide any evidence of alterations in genes involved in collagen expression. In SHRs with established hypertension, genes involved in cell death and lipid metabolism were upregulated and those involved in ion homeostasis and neurotransmitter receptor mechanisms were downregulated. This study shows that SHRs display a chronic

hypoxic state, which cannot exclusively be explained by hypertension (Ritz et al., 2012).

The number of studies carrying out microarray examination using the SHRSP model is also quite limited, with many of them examining differential gene expression in comparison to SHR. A key study carried out by Iwanaga et al., aimed to examine alterations in gene expression in vessels from SHRSP, which showed signs of remodelling and BBB leakage (Iwanaga et al., 2008). They manually micro-dissected vessels from the hippocampus and isolated a key gene altered in expression. Osteopontin gene was found to be increased in the SHRSP when compared to WKY. Osteopontin is an argine-glycine-aspartatic-acid-containing adhesion glycoprotein, which is known to interact with integrins and CD44 receptors. It has also been shown to be phenotypically similar to contractile smooth muscle cells and has been found to be expressed on perivascular macrophages and microglia. Other studies have found alterations in gene expression of brain derived neutrophic factor receptor (TrkB), MAPK/kinase pathway, including c-Jun NH(2)-terminal kinase (JNK), protein kinase B (AKT) and phosphoinositide -3- kinase (PI3K), many of which are involved in eNOS expression in 8 week old SHRSPs compared to SHRs (Fornage et al., 2003). At this time point, the SHRSPs have evidence of more severe vascular remodelling, when compared to SHRs and these genes may be play a crucial role in vascular remodelling. Overall, the above studies point towards alterations in endothelial signalling and inflammatory genes in these models of hypertension.

1.5.3. Animal models of hypertension and white matter alterations

As addressed previously, evidence in humans indicates that WML may be a sign of hypertensive induced structural alterations within the brain. In general, the number of studies providing evidence of WM alterations in the SHR and SHRSP models is quite limited. An early histological study by Hazama et al., described structural alterations within the WM of the SHRs as small cortical and subcortical areas of WM edema, gliosis and cystic formations, which were not observed in normotensive WKY (Hazama et al., 1977). More recent studies using immunohistochemistry to examine WM integrity, have reported that at around 20 weeks of age, SHR exhibit a loss of hippocampal WM, which progresses with age to a marked loss of grey matter associated with gliosis (Sabbatini et al., 2000). In turn, a study by Lin et al., examined several cortical and subcortical regions in the brains of 12 week and 20 week old SHRs but only found subtle WM loss within the striatum (Lin et al., 2001). However, the previous studies have examined overall structural integrity using histology or the expression of specific proteins to examine white matter integrity. Yang et al., took advantage of recent advances in in-vivo imaging combined with immunohistological techniques and carried out an extensive analysis of the structural integrity of the brain in SHRs. Overall, analysing 4 month old SHRs they found that in comparison to normotensive controls the WM was only modestly altered. This was quantified by an increase in T2 weighted MRI in the corpus callosum and internal capsule, which correlated to a subtle decrease in MBP (Yang et al., 2011).

In the SHRSP model of malignant hypertension, there is stronger evidence of WM alterations throughout several regions of the brain but it is possible this is more of a feature of stroke than hypertension. Ogata et al., carried out initial crude histological characterisation of WM integrity using serial sections through multiple regions of the brain in SHRSPs of varying ages. They observed, with increased duration of hypertension, areas of rarefied and cystic cortical WM, associated with remodelled and often occluded vessels (Ogata et al., 1981). Following on from these observations, Fredriksson et al., used EM to analyse the ultrastuctural integrity of WM in 8 month old SHRSPs. They frequently observed 'spongy' and 'cystic' tissue destruction in the cerebral cortex and basal ganglia, with loosening of the WM fibres. This study also found evidence of cystic lesions within the grey matter. Importantly, both white and grey matter lesions where found to be associated with alterations to the vasculature (Fredriksson et al., 1988). Chue et al., carried out an immunohistochemical assessment of WM alterations in the SHRSP and found that the subcortical WM lesions observed were associated with gliosis and proliferation of inflammatory cells. Similar results were found in 20 week old SHRSPs with marked WM loss in the corpus callosum, anterior commissure, internal capsule and caudate putamen, which was also associated with vascular remodelling and inflammation. Interestingly, a recent study examining WM integrity in 10 month old SHRSPs, without any evidence of stroke symptoms found no evidence of WM alterations even though there was evidence of vascular thickening (Brittain et al., 2012). Thus, it is unclear as to whether hypertension alone in the SHR and SHRSP models is sufficient to alter WM integrity.

1.5.4. Animal models of hypertension and cognitive function

In humans, the effect of hypertension on cognitive function is difficult to study in isolation due to the coexistence with multiple vascular risk factors and the various treatment regimes. In turn, the use of cognitive assessments to replicate the key areas of cognitive function affected in humans, can also pose a problem in animal models. The SHR model has been the most widely assessed due to its decreased severity of hypertension, in comparison to SHRSPs and their ability to carry out tasks at an older age. However, the first behavioural characteristic studies were limited to a small set of avoidance based paradigms available. One of the first studies was carried out by Hect et al., using a basic conditional avoidance task in 8-50 week old SHRs and WKYs. They found that in general the SHRs performed poorer than WKYs at varying ages (Hecht et al., 1978) but, other studies using similar tasks have found that SHRs have superior performance versus WKY (Sutterer et al., 1980), (Boller et al., 1977; Campbell and Di Cara, 1977). These findings were in agreement with the hypothesis suggested by Santinder et al., that strains selectively bred for a physiological feature may differ behaviourally by central arousal, which in turn may impair task performance (Satinder, 1977).

The design of the radial arm maze (Olton and Samuelson, 1976) and the Morris water maze (Morris, 1981), revolutionised the ability to assess more specific behavioural alterations, which can be compared to humans' abilities, without the use of painful stressors. Even with more sensitive measurements of cognitive function, performance issues between the WKY and SHR models still exist making it difficult to decipher the relationship between hypertension and cognitive function. Mori et al., used the radial arm maze to examine spatial reference and working memory in

young (3-4 month old) and aged (16-17 month old) SHRs compared to age matched WKYs. They found that hypertension per se, in the young SHRs did not cause impairment in performance when compared to WKY. Overall, in both strains age was found to cause a decrease in cognitive performance, with hypertension thought to exacerbate the impairment in SHRs (Mori et al., 1995). These results are in agreement with an earlier study carried out by Wyss et al., comparing 3 to 12 month old SHRs to normotensive Sprague Dawley strain and additionally SHRs on antihypertensive treatment (Wyss et al., 1992). They found that cognition was impaired in the SHRs compared to Sprague Dawley rats at 12 months of age and that anti-hypertensive treatment improved cognitive function. They also reported that the 3 month old SHRs showed superior performance when compared to Sprague Dawley rats (Wyss et al., 1992). On the other hand, Nakamura-palacios et al., carried out the radial arm maze test with a protocol which also assessed long term memory in 3 month old SHRs and WKYs. They found that SHRs performed worse than normotensive WKYs and this impairment was more apparent when assessing long term memory (Nakamura-Palacios et al., 1996). Similarly impaired radial arm maze performance was reported in SHRs aged 3 to 9 months, when compared to WKYs (Hernandez et al., 2003).

Other forms of cognitive testing have been used to examine cognitive function in the SHRs, such as the Morris water maze. Gattu et al., examined both spatial and reversal memory in 4 week old pre-hypertensive SHRs and in those with established hypertension at 12 weeks of age, compared to age-matched normotensive WKYs and wistar rats. It was reported that prior to the development of hypertension, SHRs exhibit impaired reversal memory but not spatial reference memory, when

compared to normotensive controls. When examining SHRs with established hypertension they found evidence of impaired function in both spatial reference and reversal memory (Gattu et al., 1997a; Gattu et al., 1997b). A follow up study in SHRs aged 15 months was carried out and they were found to have impaired performance when compared to WKY (Terry Jr et al., 2000). On the other hand, a study by Widy-Tyszkiewicz et al., examined spatial reference memory in 12 week old SHR and WKY using the Morris water maze and found SHRs to have superior performance, suggestive of cognitive superiority (Widy-Tyszkiewicz et al., 1993). Overall in the SHR model there is evidence of cognitive impairment but discrepancies in the performance of normotensive WKY prevent these findings from being robust.

The SHRSPs have been found to exhibit behavioural abnormalities such as inattention, hyperactivity and impulsivity prior to the development of hypertension (Ueno et al., 2002). This makes the SHRSP a commonly used model for attention deficit - hyperactivity disorder (AD/HD) research, and may suggest some limitations in their use for hypertensive research, with overall cognitive data being quite sparse in this model. In general, the SHRSP have been found to display no overt neurological deficit prior to the development of stroke (Jiwa et al., 2010), with impairments reported in passive avoidance behaviour at 30 weeks of age (Kimura et al., 2000). Similar deficits in passive avoidance and Y-maze paradigms have been reported (Kimura et al., 2000; Minami et al., 1997; Sugimachi et al., 1994; Togashi et al., 1996). Spatial reference memory has also been examined in the SHRSP models. Interestingly, a study comparing the performance of 15 week old SHRSPs, SHRs and WKYs using two types of water maze; the Biel and Morris, found that the

SHRSP model had impaired performance compared to WKYs, whereas SHRs did not. They also observed that the SHRSPs showed impaired swim durability during the Biel maze (Matsuo, 2007). Overall, it is not yet known whether hypertension in both the SHR and SHRSP models leads to alterations in cognitive function.

1.6. Cyp1a1 Ren2 inducible hypertensive rat model

Invaluable as they have been, the SHR and SHRSP models have their limitations, which have produced gaps within the understanding of the mechanisms of hypertension. No animal model recapitulating a human condition is perfect and often a true understanding of the disease requires the investigation of several animal models. To model the effect of hypertension on the structural integrity of the cerebrovasculature, WM and cognitive function, this thesis used the Cyp1a1 Ren2 rat model, which allows the expression of hypertension in a controlled, sustained manner without the need for surgical intervention, with genetically matched litter-mate controls and the ability to map hypertensive mechanisms in aged animals, comparative to ages at which hypertension occurs in humans.

Cyp1a1 Ren2 rat model is an inducible hypertensive transgenic model generated by the insertion of a mouse Ren2 renin gene fused to cytochrome P450 1a1 (Cyp1a1) promoter on the background strain of the Fischer 344 rat (Kantachuvesiri et al., 2001). Expression of this transgene is induced by the addition on an aryl hydrocarbon; Indole-3-carbinol (I3C), allowing induction of the Cyp1a1 promoter via the aryl hydrocarbon receptor leading to the primarily hepatic expression of renin, as shown in Figure 1.9 (Fujii-Kuriyama et al., 1995; Jellinck et al., 1993; Loub

et al., 1975). These transgenic rats are born normotensive since the promoter is not constitutively expressed (Kantachuvesiri et al., 2001).

The level and duration of hypertension is tightly controlled by the addition of I3C, which is a naturally occurring agent found in cruciferous vegetables (Loub et al., 1975). The short half-life of I3C allows for the reversal of hypertension in this model, with early studies showing blood pressure can return to normotensive values within 72 hours of I3C removal. Critically non transgenic animals, which are given I3C even at very high doses do not become hypertensive (Kantachuvesiri et al., 2001).

Early characterisation studies of this model demonstrated two methods of hypertension induction, by means of gastric gavage or dietary induction (Kantachuvesiri et al., 2001). I3C can be mixed with standard powdered rat chow, allowing transgenic induction in a non-surgical and non-stressful manner. Dietary induced I3C produces a dose-dependent increase in blood pressure, with studies showing a dose of 0.3% I3C leads to malignant hypertension, whereas 0.15% I3C leads to a milder form of hypertension (Mitchell et al., 2006). Transgenic animals induced with 0.3% have rapidly increased blood pressure by 1 day, whereas those induced by 0.15% develop increased blood pressure within 2 weeks (Mitchell et al., 2006).

The majority of studies in this model have focused on the periphery providing evidence of increased levels of plasma renin, 48 hours post induction (Kantachuvesiri et al., 2001). These studies have shown target organ damage to the heart and kidneys. Within 4 days of the high 0.3% dose of I3C there was fibrinoid

necrosis of small arteries in the mesentery and heart, which lead to microinfarction in both the left and right ventricles and evidence of eutrophic remodelling to the mesenteric vasculature (Kantachuvesiri et al., 2001). The kidneys showed evidence of myointimal hyperplasia, tubular dilation, glomerulosclerosis, tubulointerstitial inflammation and proliferation (Graciano et al., 2007; Kantachuvesiri et al., 2001; Mitchell et al., 2006). A crude characterisation of the brain provided no evidence of haemorrhage, infarction or gross vascular pathology (Kantachuvesiri et al., 2001). Therefore, a robust characterisation of the structural integrity of the brain with hypertension has not yet been established in this model. This model allows hypertension to be studied in a controlled and sustained fashion, without the need for surgical intervention and comparisons can be made with litter-mate transgenic normotensive controls.

1.7. Summary

Overall, previous studies in both humans and animal models of hypertension have allowed for an insight as to the consequence of chronic high blood pressure on the structural and functional integrity of the brain. However, the mechanisms which underlie these associations are not yet fully understood. In humans this is partly due to the frequent coexistence of hypertension with other vascular risks factors and the inability to model hypertension at a set duration, in a large untreated population of similar age and severity. The SHR and SHRSP models have provided invaluable knowledge as to the effect of hypertension on the cerebrovasculature, but it is unclear the exact influence of hypertension alone and not genetic predisposition. The studies outlined in this thesis sought to investigate whether hypertension in isolation leads to

impaired structural integrity of cerebrovasculature and WM and whether hypertension in isolation is a sufficient insult to impair cognitive function. These features were investigated using an inducible hypertensive rat model, in which the severity, duration and onset of hypertension were tightly controlled, and findings compared to genetically matched littermate normotensive controls. Additionally, the use of genetically matched controls will also allow for the profiling of differentially expressed genes related to vascular remodelling with hypertension.

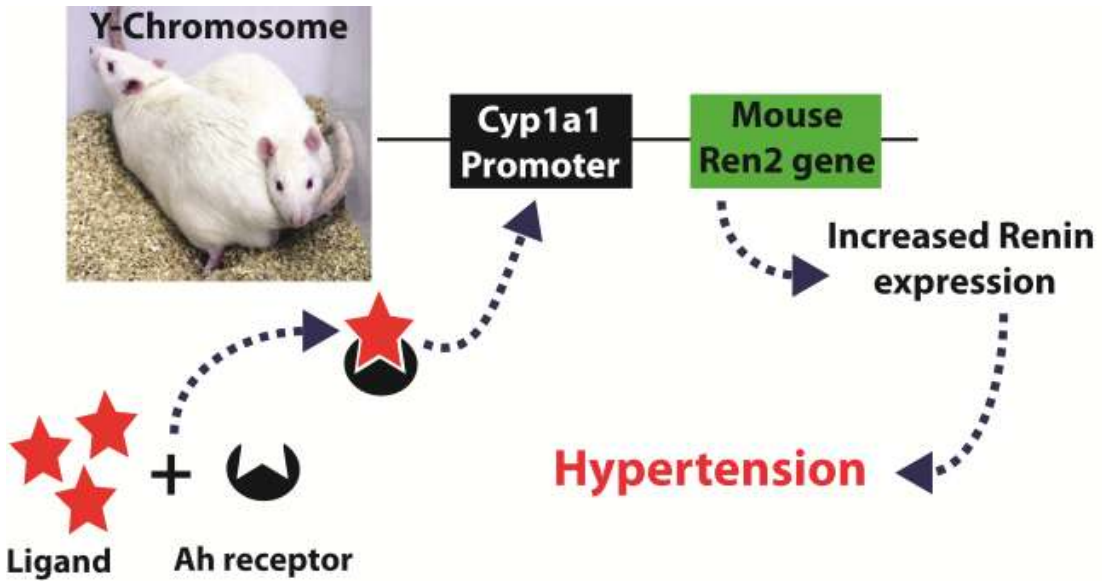

Figure 1.8: Induction of hypertension in the Cyp1a1 Ren2 rat model
Hypertension is induced in the Cyp1a1 Ren2 rat model by the expression of the Cyp1a1 promoter. Cyp1a1 is not constitutively expressed but is activated upon exposure to various aryl hydrocarbons. In this study Indole-3-carbionol represented as 'Ligand' in the image, acts via the aryl hydrocarbon receptor (Ah receptor), to induce the expression of the Cyp1a1 promoter, which then allows the expression of the mouse ren 2 gene causing increased expression of renin and ultimately increased blood pressure.

1.8. Thesis hypothesis

It was hypothesised that sustained hypertension will lead to alterations to the structural integrity of the cerebrovasculature, WM and associated alterations in gene expression within the young and aged brain and that hypertension will be associated with impairments to cognitive function.

1.8.1. Experimental chapters hypotheses and aims

- Hypothesis: Hypertension will lead to vascular alterations within the subcortex of the young and aged brain consisting of remodelling of the basement membrane, loss of BBB integrity and altered endothelial signalling.

  Aim: To investigate the impact of hypertension on the structural integrity of the vasculature - vascular width and density, endothelial signalling, endothelial blood-brain barrier integrity and the inflammatory response. The differences in structural integrity of the vasculature were investigated in the young and aged brain of normotensive and hypertensive animals.

- Hypothesis: Hypertension will lead to alterations in gene expression in particular those related to vascular remodelling, endothelial signalling and inflammation.

  Aim: To investigate the effect of hypertension on gene expression and functional pathways using microarray technology in the young brain and in reference to structural alterations observed.

- Hypothesis: Hypertension will lead to alterations to the integrity of white matter in the young and aged brain.

  Aim: To investigate the impact of varying durations of hypertension on the structural integrity of WM with an examination of myelin, oligodendrocytes, axons and microglial response, in specific regions of the young and aged brain.

- Hypothesis: Hypertension will lead to impaired cognitive function in spatial reference and working memory

  Aim: To investigate the effect of hypertension on cognitive function, examining spatial reference and working memory in hypertensive versus normotensive rats.

2. Materials and methods

2.1. Animals

Studies within this thesis chose to examine the integrity of the brain using the Cyp1a1 Ren2 transgenic rat model. This model allows the induction of hypertension in a controlled, gradual and sustained manner, without any surgical intervention. In turn, all comparisons made in this model can be compared to genetically matched litter-mate controls.

The Cyp1a1 Ren2 transgenic rat model is generated by the insertion of a mouse ren-2 gene fused to the cytochrome P450 1a1 (Cyp1a1) promoter on the Fischer 344 background, which was created by Prof. John Mullins. Characterisation studies described that these transgenic rats were originally created by the isolation of the rat Cyp1a1 promoter through *Not*I-*Sal*I double digest of pAhIR1-LacZ, yielding an 11.5 kilobase fragment, which was placed upstream of the Ren-2 cDNA and SV40 poly(A) signal in pBluescript SK2(+). *Not*I-*Bss*HII digestion was then used to excise the injection fragment, which was introduced into single-cell embryos of Fischer F344 by microinjections and was found to be integrated on the Y chromosome (Kantachuvesiri et al., 2001). Transgenic rats used for studies within this thesis were bred from a male and female transgenic rat obtained from Prof. John Mullins at the University of Edinburgh. Harems were set up in which the one male was housed with 3 female rats, which were removed once pregnant. As the transgene is Y chromosome specific, only male transgenic rats were used for the study of hypertension. As introduced in section 1.5 (Figure 1.9), mouse renin gene expression is activated by the addition of the aryl hydrocarbon I3C allowing the activation of the Cyp1a1 promoter.

2.1.1. Indole-3-Carbinol administration

Hypertension was modelled in the Cyp1a1 Ren2 rat model by dietary induction of I3C based on the protocol devised in previous characterisation studies (Kantachuversiri et al., 2001). All animals were housed in 12 hour light/dark cycle and had access to food and water ad libitum, except from during experimental testing, when food intake was controlled and weight monitored (throughout the duration of the study). Experiments were conducted under approval of the UK Home office licence and regulations (Specific procedures Act 1986). Animals were either fed a control diet containing a mashed version of standard commercial rat chow (40g per animal) (Special diet services, Witham, Essex) or a hypertensive diet with 0.15% of I3C, mixed into a mashed version standard chow (40g per animal). Each animal was given a glass jar containing either normotensive or hypertensive diet, and this was replaced with fresh diet daily. Daily monitoring throughout the study found that the animals ate the majority of the diet each day, with only a small amount left over.

2.1.2. Study cohorts of animals

Experiments were conducted in both young and aged Cyp1a1 Ren2 rats, dietary induced for different durations. Studies were conducted on young animals aged 3 to 4 months prior to dietary induction, induced for a period of either 4 or 6 months and referred to throughout this thesis as the young 4- or 6-month cohort respectively. Initially the 4 month time point was chosen as several of the studies in SHRs reported cerebrovascular alterations occurring at 3 months of age (Knox et al., 1980; Ueno et al., 2004a). Since blood pressure rises slowly in these animals it was decided to dietary induce for a period of 4 months. The 6 month period on the other

hand was chosen as the animals began to show some subtle features of possible progression towards malignant hypertension, such as piloerection and lethargy thus, the study was terminated prior to the animals becoming ill. The young 4-month cohort was separated into two groups; n=12 normotensive and n=16 hypertensive animals, which were processed for immunohistochemical analysis and n=12 normotensive and n=12 hypertensive animals for cognitive assessment. During dietary induction the young 4-month cohort processed for immunohistochemistry were housed in Little France animal unit, whilst the young 4-month cohort designated towards behavioural assessment were housed in George Square animal unit thoughout the duration of the study. The young 6-month cohort was separated into two groups run concurrently; n=13 normotensive and n=13 hypertensive for immunohistochemical assessment and n=8 normotensive and n=8 hypertensive animals for biochemical and gene assessments. These animals were originally bred in Little France animal unit but were transferred to George Square animal unit prior to the start of the study and were housed there until study termination. The study also ran an aged cohort of n=10 normotensive and n=12 hypertensive animals, which were allowed to age naturally to around 14-15 months of age then dietary induced for a period of 4 months and referred to as the aged 4-month cohort. These animals were bred in the George square animal unit but then moved to Little France animal unit to age and for the duration of the study.

2.1.3. Animal monitoring

2.1.3.1. Weight

Throughout the duration of the study weight measurements were taken to ensure animals weight did not drop below 20% of their pre-study weight. Overall

weight measurements can be found in Figure 2.1. During the experimental period the cages of animals were monitored daily to ensure consumption of the diet.

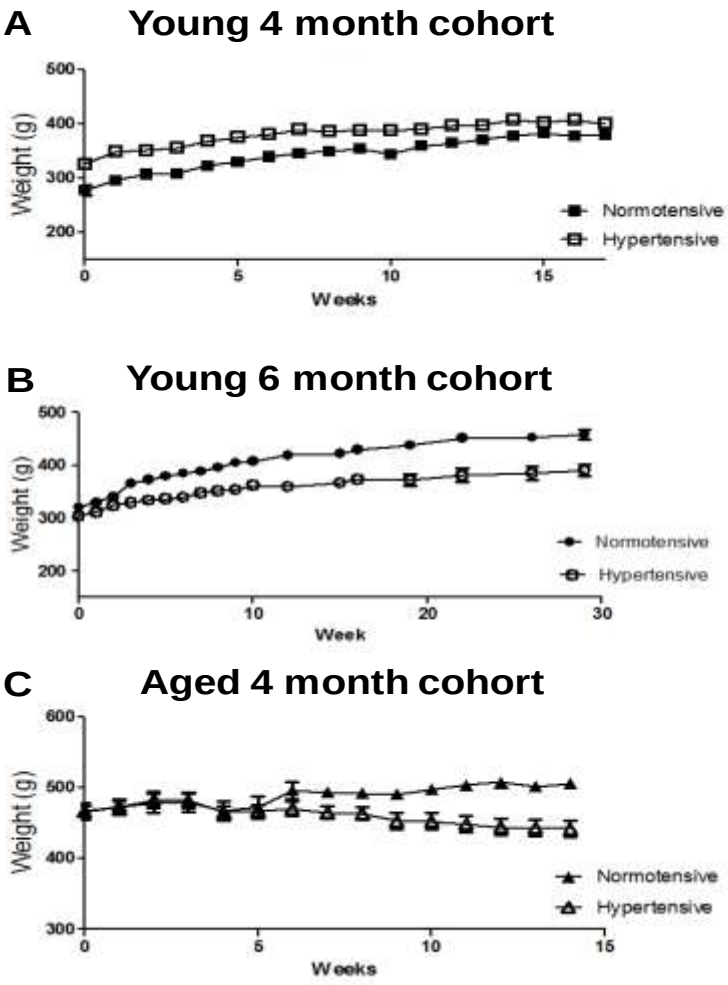

Figure 2.1: Weight measurements

Representative graphs of weight measurements for each cohort throughout the duration of the study. Dietary induction began at week 1 with statistical significance of weight readings between groups examined by repeated measures ANOVA. (A) Weight measurements for animals from the young 4-month cohort. All animal continued to gain weight within the first few weeks post dietary induction. There was no significant difference in weight between the two treatment groups p= 0.153 but there was a significant difference between weight over time p<0.001. (B) Weight measurements for the young 6-month cohort provided evidence of weight gain after initial weeks of dietary induction. However, there was a significant difference in weight between the two treatment groups p=0.006 and over time p<0.001. (C) In the aged 4-month cohort animals gained weight in the first few weeks after dietary induction but by week 6 there is a significant difference between the two treatment groups p=0.004 and over time p<0.001. Graphs show mean ± SEM.

2.1.3.2. Blood pressure

Blood pressure was monitored using tail-cuff plethysmography (LE5002; Panlab, Harvard appliances). All rats were trained for 4 weeks prior to dietary induction with blood pressure measurements taken throughout the duration of the study.

Within each blood pressure recording session the order of which animals were monitored was randomised. Prior to blood pressure recordings animals were habituated in the blood pressure room for 20 minutes, then one animal at a time was placed in an incubator of ambient temperature (no more than 35°C, no less than 30°C) for 5 minutes. During blood pressure recordings rats were restrained in a cylinder plexiglass chamber with tail access, under a heater maintaining a temperature of around 35°C. Animals were habituated in the restraint and tail-cuff prior to blood pressure recordings for 5 minutes. Blood pressure was measured using an electro-sphygmomanometer and pneumatic pulse transducer. Tail blood was occluded by an 11 mm tubular cuff that was inflated by an automatic cycling cuff pump, with automated deflation of the cuff at appropriate pressures calculated from each animal's initial systolic reading. NIBPchart (Panlab, Harvard appliances) software was used to track recordings ensuring a steady state pulse was achieved with the average of three recordings taken per animal. Measurements were excluded if the animal moved during recordings. Within a session animals were excluded if they failed to habituate and were retested a few hours later. Throughout this thesis blood pressure is reported by systolic blood pressure measurements due to a close correlation between indirect tail cuff measurements and measurements obtained using femoral or carotid catheters (Bunag and Butterfield, 1982).

2.2. Tissue processing

2.2.1. Perfusion fixation

Animals were terminated by transcardiac perfusion carried out by Dr P Holland. Anaesthesia was induced with 5% isoflurane in an anaesthetic chamber and then maintained at approximately 2% isoflurane. Transcardiac perfusion was carried out by opening of the thoracic cavity exposing the diaphragm. The diaphragm was cut through, exposing the heart, which was cleared of connective tissue. Once the connective tissue was cleared a needle was inserted and clamped into place in the left ventricle and a second incision was made in the right ventricle to allow drainage of blood. Perfusion rate was matched to each animal's end stage blood pressure measurement to provide optimal perfusion. 200mL of heparinised 0.9% saline in 0.1% phosphate buffer was infused until the solutions ran clear. For biochemical studies, rats were perfused by heparinised saline only and the brains were then removed from the skull and snap frozen in liquid nitrogen. For immunohistochemistry rats were perfused with 4% paraformaldehyde in phosphate buffer (pH=7.4), decapitated and the whole head placed in 4% paraformaldehyde for 2 hours. Brains were removed from the skull and kept in 4% paraformaldehyde at 4˚C for 48 hours following 24 hours in phosphate buffer solution. Heart, mesenteries and kidneys were also collected and postfixed by the same method.

2.2.2. Paraffin embedding

Brains perfused for immunohistochemistry were cut into 3mm blocks using a rat matrix. Coronal slices were placed in plastic embedding cassettes, dehydrated by

successive concentrations of alcohols and paraffin embedded (table 2.1). Both dehydration and embedding were carried out on an automatic tissue processor; Tissue Tek VIP 2 (Sakura Fintek Europe, Netherlands). Tissue for the young 4-month and 6-month cohorts was processed using the automatic tissue processor in the Hugh Robson Building whereas; the tissue from the Aged 4-month cohort was processed using the automatic tissue processor in the Queen's medical research institute facility. Post processing and embedding, the blocks were cut at 6µm using a microtome, with assistance from Miss Fiona Scott for the aged cohort. Sections were mounted onto charged slides (Super frost Plus, VWR International, Butterworth, UK) and allowed to dry on a hot plate for 2 hours before storage in plastic containers. The heart, mesenteries and kidneys were also collected and embedded by the same method as the brain sections. The heart and kidneys were sectioned in half prior to embedding but the mesenteries remained whole.

2.2.3. Immunohistochemistry

Brain sections used for immunohistochemical analysis Figure 2.2 (-3.14mm from Bregma to -5.14mm) (Paxinos and Watson, 1998) and for each immunohistochemical analyses, there were two brain sections mounted on each slide, both of which were analysed and an average value taken. All analyses were carried out blind to the experimenter. Each of the 3 cohorts used for immunohistochemistry were stained separately each on individual runs. Sections were deparaffinised at 60°C, immersed in xylene and rehydration in a series of graded alcohol solutions. 3% H_2O_2 was used to block endogenous peroxidase activity for 30 minutes proceeded by washing in running water for 10 minutes. When further antibody penetration was

required antigen retrieval was performed (Table 2.2). Sections were then washed in phosphate buffered saline (PBS) for 10 minutes following incubation in a blocking solution of 5% bovine serum, 10% normal serum (specific for the species the antibody is raised in blocking nonspecific activity) and PBS for 1 hour at room temperature. Primary antibody solution was added to the blocking solution; sections were drained of block and incubated overnight at 4°C.

Day two of the immunohistochemical staining, sections were allowed to come to room temperature for 10 minutes, and then washed twice in PBS for 10 minutes. The appropriate secondary antibody solution was made at a dilution of 1:100 with PBS and applied to the sections that were then incubated for 1 hour at room temperature. After incubation the secondary was drained off and sections were washed twice in PBS for 10 minutes. For amplification, sections were incubated in avidin-biotin-peroxidase complex solution (Vector Labs, Peterborough, UK) for 1 hour at room temperature then washed twice in PBS for 10 minutes. Diaminobenzidine (DAB) (Vector Labs, Peterborough, UK), used for antibody labelling was applied and developed for 3 minutes, then washed under running water for 10 minutes. Sections were dehydrated in a series of alcohol solutions followed by xylene and then coverslipped with DPX mounting medium (Thermo Fisher, Loughborough, UK). When required counterstaining with haemotoxylin or periodic acid Schiff's (PAS) was applied before the dehydration step. Prior to analysing all sections were screened including the negative to assess the success of staining.

Details of each primary antibody used within this thesis, including their corresponding secondary antibody and antigen retrieval can be found in Table 2.2. Each antibody concentration was devised by a series of optimisations at several

different antibody concentrations and retrieval methods, using tissue from the same cohort of animals which staining was to be carried out on. In addition antibody specificity was controlled, as each experiment included one blank slide for each cohort that underwent all the immunohistological treatments apart from the addition of the primary antibody allowing the reader to decipher between non specific and specific staining.

Solution	Duration (minutes)	Temperature (˚C)
50% Ethanol	**60**	**35**
80% Ethanol	**60**	**35**
95% Ethanol	**60**	**35**
100% Ethanol	**60**	**35**
100% Ethanol	**60**	**35**
100% Ethanol	**60**	**35**
100% Ethanol	**60**	**35**
Xylene	**3 x 60**	**35**
Paraffin wax	**3 x 60**	**60**
Paraffin wax	**Set for a minimum of 60**	**60**

Table 2.1: Tissue processing
Prior to paraffin embedding the tissue was dehydrated by several washes with increasing concentrations of alcohol followed by a final wash in xylene.

Primary antibody	Supplier	Dilution	Antigen retrieval	Normal serum	Secondary antibody
Collagen IV (2A)	Fitzgerald, Massachusetts, USA	1:7500	Antigen retrieval unit *(10 minutes in 0.01mol/L citric acid buffer, followed by 10 minutes in 0.02 mol/L Proteinase K buffer)*	Goat	Anti rabbit
α-Smooth muscle actin	Millipore, Watford, UK	1:100	Antigen retrieval unit *(5 minutes in 0.01mol/L citric acid, followed by 10 minutes in 0.02 mol/L Proteinase K buffer)*	Horse	Anti mouse
Claudin-5	Invitrogen, Califonia, USA	1:100	Antigen retrieval unit *(10 minutes in 0.01mol/L citric acid and left for 10 minutes to cool)*	Horse	Anti mouse
ZO-1	Invitrogen, Califonia, USA	1:50	Antigen retrieval (30minutes in 0.01mol/L protease buffer at 37˚C)	Goat	Anti rabbit
eNOS	BD Biosciences, California, USA	1:150	N/A	Goat	Anti rabbit
MBP	Millipore, Watford, UK	1:5000	N/A	Rabbit	Anti rat
APP	Millipore, Watford, UK	1:10000	Antigen retrieval unit *(10 minutes in 0.01mol/L citric acid buffer)*	Horse	Anti mouse
Iba-1	Menarini, Berkshire, UK	1:1000	N/A	Goat	Anti rabbit
CC1 (APC, Ab-7)	Novus Biologicals, Cambridge, UK	1:150	Antigen retrieval unit *(10 minutes in 0.01mol/L citric acid buffer)*	Horse	Anti mouse

Table 2.2: Details of antibodies used in immunohistochemical assessment
Details the antibodies use for immunohistochemical assessment in this thesis including manufacturer, dilution, antigen retrieval, normal serum and secondary antibody used.

2.2.4. Vascular integrity

Several markers were used to analyse various components of the cerebrovasculature. All vessels examined were from the subcortical region of the brain as can be seen in figure 2.2 and were averaged for two adjacent sections. Each of the analyses described below required images to be taken using a light microscope. This was carried out separately for each cohort and light settings were set specifically for each cohort.

2.2.4.1. Quantification of the cerebral vasculature

The cerebral vasculature integrity was analysed through immunostaining the vasculature for α-smooth muscle actin (SMA) and collagen IV (specific for subunit 2A). SMA was quantified by examining the density through percentage area stained measurements using Image J (U.S. National Institutes of Health, Bethesda, USA). For each cohort, 2 images per hemisphere at x40 magnification were taken using QImaging QICAM MicroPulisher 3.3 camera (QImaging, Surrey, BC, Canada) connected to a Olympus BX51 microscope (Olympus UK, Southendon-Sea, UK) with light settings maintained constant. Firstly, the region of choice was identified at the lowest magnification and then the magnification was increased. The first subcortical image taken used the edge of the fimbria and internal capsule as land marks and the second used the area underneath the dentate gyrus prior to third ventricle as a land mark, with the same process repeated for the opposite hemisphere. This method was used throughout and as much as possible the same location was used for each section from each animal. Density was calculated by setting a threshold value for positive staining, which remained the same for each animal within a cohort.

The threshold value was set by the optimum value, allowing for identification of positive staining and eradication of background. This value was calculated for each animal within a cohort with the average threshold value used for the whole group. As with other techniques reproducibility was established by the triple repetition of the quantification of 3 randomly selected animals with values achieved between 10% of the median for both density measurements and thresholding.

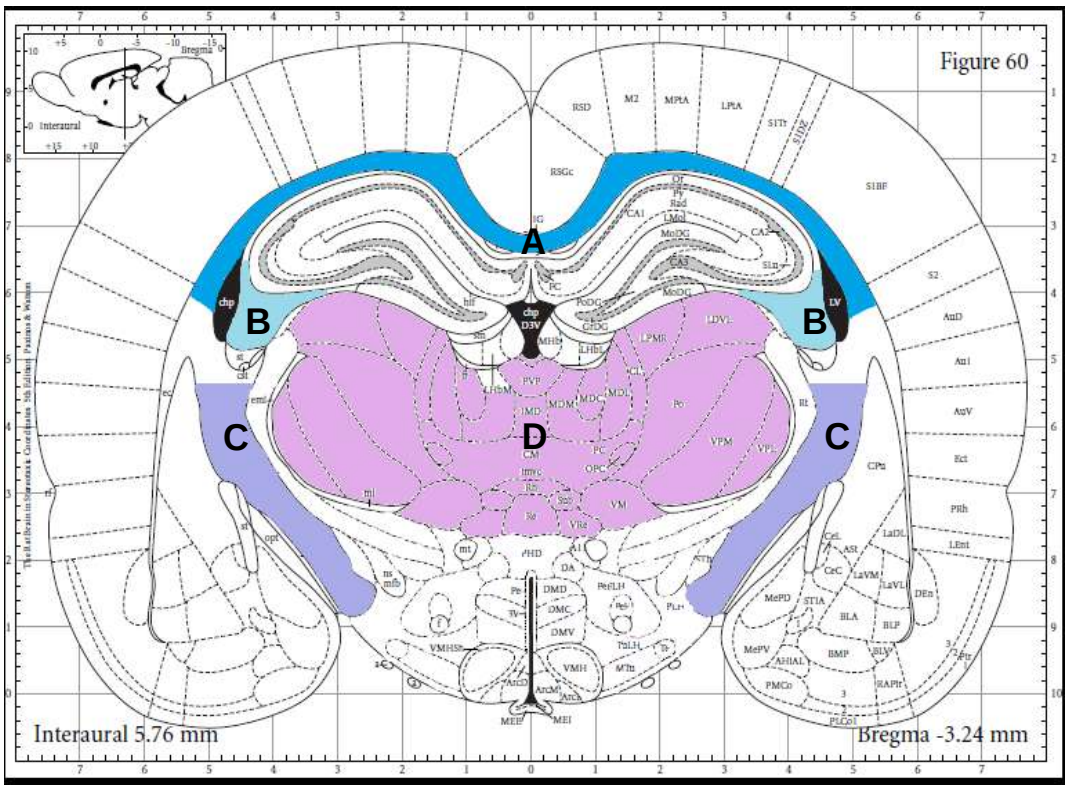

Figure 2.2: Regions analysed throughout this thesis.
Image adapted from Paxinos and Watson brain atlas representative of regions analysed for immunohistochemistry and subcortical thalamic region dissected for microarray analysis (Paxinos and Watson 1998). (A) Corpus callosum, (B) fimbria, (C) internal capsule and (D) subcortical thalamic region were all analysed for assessment of white matter integrity. (D) subcortical thalamic region was analysed for vascular integrity, white matter integrity and microarray gene alterations.

An antibody specific for collagen IV (2A), a marker of the basement membranes was used as a global marker of the vasculature. Firstly using QImaging QICAM Fast 1394 camera (QImaging, Surrey, BC, Canada) connected to an Olympus BX51 microscope (Olympus UK, Southendon-Sea, UK), 2 images per hemisphere were taken at x200 magnification as described for smooth muscle actin. Collagen IV labelled vasculature in both a longitudinal and cross sectional orientation. Image J was used to calculate the vascular width of vessels in the longitudinal orientation. A grid was placed on the image and only vessels which crossed the grid were measured, with more than 20 vessel measurements taken per animal.

As described for SMA, the density of Collagen IV staining was calculated allowing for an indication of overall Collagen IV levels. Additionally, the number of vessels within 0.25mm^2 area of the subcortex was calculated, by applying a grid onto images and counting the number of vessels which crossed lines on the grid, ensuring that the vessels measured were chosen at random.

2.2.4.2. Quantification of endothelial Nitric Oxide Synthase labelled vessels

Endothelial signalling was investigated by the expression of endothelial specific antibody for endothelial nitric oxide synthase (eNOS). Localisation of eNOS with the vasculature was validated by haemotoxylin counterstain and could be clearly identified by brown DAB staining outlining the vasculature. The number of vessels immunopositive for eNOS were counted within a 1mm^2 area of the subcortex. Images were taken as described for smooth muscle actin at x100 magnification within the subcortex using QImaging QICAM Fast 1394 camera (QImaging, Surrey,

BC, Canada) connected to an Olympus BX51 microscope (Olympus UK, Southendon-Sea, UK) and the number of eNOS expressing vessels counted using Image J (U.S. National Institutes of Health, Bethesda, USA).

2.2.4.3. Quantification of endothelial tight junctions

The integrity of tight junctions was analysed using claudin-5 in which both the intensity and density of staining were calculated by taking relative optical density (ROD) measurements and percentage area stained respectively using Image J (U.S. National Institutes of Health, Bethesda, USA). In the young 4- and 6- month cohort both imaging and quantification was carried out using QImaging QICAM Fast 1394 camera (QImaging, Surrey, BC, Canada) connected to a Leica DMR microscope (Leica Microsystems UK, Milton Keynes, UK) calculated by MCID image analysis system (MCID 7.0, InterFocus Imaging Ltd, Cambridge, UK). Vascular ROD of claudin-5 was calculated within the subcortical thalamic region at a magnification of x400 and within an area 0.2mm^2 per hemisphere by manually drawing around more than 20 vessels per animal. MCID was used for this experiment as it allows the user to manually draw around each vessel separately at the same time, yielding a reading for each individual vessel and also an average. As with collagen IV analysis, a grid was applied and only vessels which crossed the grid were analysed and an average reading was calculated per animal. However, the aged 4-month cohort was analysed using different software as the MCID system had broken down prior to analysis of the aged cohort but the method was kept as similar to the young cohorts as possible. In the aged 4-month cohort ROD was measured from images taken using QImaging QICAM Fast 1394 camera (QImaging, Surrey, BC, Canada) connected to an

Olympus BX51 microscope (Olympus UK, Southendon-Sea, UK) and ROD calculated by Image J (U.S. National Institutes of Health, Bethesda, USA). The same method was used for the aged 4-month cohort, as the young by manually drawing around vessels which crossed the grid in images taken at x400 magnification within an area of $0.2mm^2$. Reproducibility was verified by repeating measurements in 3 animals from each cohort with median value less than 10%.

The density of claudin-5 staining was calculated for each cohort using Image J (U.S. National Institutes of Health, Bethesda, USA). As described previously the percentage area stained was calculated within the whole area ($0.2mm^2$) for each cohort by setting a threshold for positive staining.

For the young 6-month cohort only, the width of longitudinal vessels immunopositive for claudin-5 was calculated using the same methodology as used for Collagen IV analysis. Images were taken at x200 magnification using QImaging QICAM Fast 1394 camera (QImaging, Surrey, BC, Canada) connected to an Olympus BX51 microscope (Olympus UK, Southendon-Sea, UK) and width calculated using Image J (U.S. National Institutes of Health, Bethesda, USA). Width measurements were taken within an area $0.2mm^2$ per hemisphere and only vessels which crossed lines of the applied grid were measured.

Antibody specific for ZO-1, a tight junction association protein which tethers claudin-5 to the cytoskeleton was used to further analyse tight junction integrity in the young 6-month cohort only. Subcortical images were taken at x400 magnification using QImaging QICAM Fast 1394 camera (QImaging, Surrey, BC, Canada) connected to an Olympus BX51 microscope (Olympus UK, Southendon-Sea, UK)

and percentage area stained in an area of 0.25mm^2 was calculated using Image J (U.S. National Institutes of Health, Bethesda, USA).

2.2.4.4. Microglial alterations

Ionized calcium-binding adapter molecule 1 (Iba1) staining was used to investigate the localisation of microglial cells with the surrounding vasculature. Iba1 sections were counterstained with PAS reagent labelling the basal lamina pink, clearly outlining the vasculature. Using a light microscope the presence or absence of microglial cells associated with the vasculature within the subcortical thalamic region were recorded. Microglial cells were counted to be associated with the vasculature if they were found to be surrounding or contacting the vessel wall. General region specific microglia activation was assessed by counting the number of IBA1 immunopositive cells, per defined area. In the young 4-month cohort images were taken at x250 magnification using QImaging QICAM Fast 1394 camera (QImaging, Surrey, BC, Canada), connected to a Leica DMR microscope (Leica Microsystems UK, Milton Keynes, UK). In the young 6-month and aged 4-month cohort images were taken at x200 using QImaging QICAM MicroPulisher 3.3 camera (QImaging, Surrey, BC, Canada) connected to a Olympus BX51 microscope (Olympus UK, Southendon-Sea, UK). All images were taken at a fixed light intensity for each cohort and analysed blinded to a single experimenter. Numbers of cells were counted within a fixed area per region defined by a counting grid; corpus callosum 0.2mm^2, fimbria 0.1mm^2, internal capsule 0.15mm^2 and subcortical thalamic region 0.25mm^2. To be counted, microglia had to satisfy specific criteria of having a cell body and processes. Iba1 immunopositive microglial processes without a cell body were not counted. As with ROD data, reproducibility was established by repeating

counts 3 times, for 3 animals, with median measurements less than 10% of each other. To ensure reproducibility did not deviate within a large data set, all measurements from the young 4-month cohort were carried out twice and deemed reproducible if they were less than 10% deviated from the median reading for their treatment group. In addition, prior to statistical analysis of the data, the data from each cohort was checked for outliers.

2.2.5. Haematoxylin and eosin staining

Structural integrity of the kidney and the brain were assessed using haematoxylin and eosin (H&E), with assistance from Miss Fiona Scott. Brain sections used corresponded to -3.14mm from bregma (fig.2.2) (Paxinos and Watson 1998). Both histological and immunohistochemical analyses were carried out blinded.

Sections were deparaffinised in an oven at 60°C, for 30 minutes followed by 15 minutes in xylene. Rehydration was carried out by immersion through a series of decreasing alcohol concentrations and sections were finally immersed in running tap water for 5 minutes. Sections were then placed into Shandon haematoxylin solution (ThermoFisher, Loughborough, UK) for 2 minutes then briefly rinsed in tap water. Differentiation of stain to achieve blueing of the nuclei was carried out using acid alcohol solution (1% HCl in 70% ethanol) for 8 seconds, then briefly sections were rinsed in tap water before being placed in Scott's tap water solution, (2% $MgSO_4$, 0.35% $NaHCO_3$) for 2 minutes and then placed in running tap water for another 2 minutes. Sections were placed in solution of eosin Y (ThermoFisher, Loughborough, UK) for 2 minutes, and then briefly rinsed in tap water. Dehydration was carried out in increasing concentrations of alcohol solutions starting with 70% ethanol followed

by 15 minutes in xylene. Sections were then coverslipped using DPX mounting medium (ThermoFisher, Loughborough, UK).

2.2.5.1. Kidney pathology

A gross assessment of the overall kidney integrity of the kidney was conducted using H&E, examining for the presence or absence of vascular enlargement, tubule dilation, accumulation of inflammatory cells and protein accumulation or necrosis of the glomeruli based on previous findings in the model (Graciano et al., 2007).

2.2.5.2. Structural integrity of the brain

H&E staining was used as a marker of overall structural integrity of the brain. Neuronal damage was assessed in the hippocampus, fimbria and subcortical thalamic region (Fig 2.2). The structural integrity of overall tissue morphology was assessed in the cortex, corpus callosum, fimbria, hippocampus, thalamus and internal capsule for evidence of tissue pathological alterations such as pallor in staining, microbleeds and haemorrhages. The presence or absence of eosinophilic damage to neuronal perikarya and evidence of pathological tissue alterations were recorded. Damaged neurons could be identified by the cytoplasm appearing red eosinophilic and can be readily distinguished from dark cell change (Jortner, 2006). Small areas of microbleeds or larger haemorrhages could be identified by the presence of extravasations of erythrocytes also appearing red in colour.

2.2.6. Quantification of myelin integrity

Myelin integrity was assessed in the corpus callosum, fimbria, subcortical thalamic region and internal capsule using myelin basic protein (MBP) immunostaining. Quantification of myelin damage was analysed by calculating ROD in a defined area using Image J (U.S. National Institutes of Health, Bethesda, USA). In the young 4-month cohort images were taken at x25 using QImaging QICAM Fast 1394 camera (QImaging, Surrey, BC, Canada), which was connected to a Leica DMR microscope (Leica Microsystems UK, Milton Keynes, UK). Additionally, to coincide with cognitive data, the hippocampus was also investigated within this cohort. Images were taken to encompass the entire hippocampus and the ROD was taken of the whole region. In the young 6-month and aged 4-month cohort images were taken at x40 using QImaging QICAM MicroPulisher 3.3 camera (QImaging, Surrey, BC, Canada) connected to a Olympus BX51 microscope (Olympus UK, Southendon-Sea, UK). All images were taken at a fixed light intensity for each cohort and analysed blinded to a single experimenter. ROD measurements were taken within a fixed area per region; corpus callosum 0.2mm^2, fimbria 0.1mm^2, internal capsule 0.15mm^2 and subcortical thalamic region 0.25mm^2 with measurements averaged over hemispheres. Measurements are expressed as the percentage difference from normotensive control values. In order to establish reproducibility within measurements, all measurement within three animals were carried out three times and deemed reproducible, by each measurement falling within 10% of the median for each animal. Prior to breaking the codes the data sets were plotted and any outliers, reanalysed to ensure data points were accurate.

2.2.6.1. Quantification of number of oligodendrocytes

Quantification of oligodendrocyte cell number was assessed by counting the number of adenomatous polyposis coli protein (CC1) positive cell bodies. For all cohorts images were taken at x200 within the subcortical thalamic region only using QImaging QICAM MicroPulisher 3.3 camera (QImaging, Surrey, BC, Canada) connected to a Olympus BX51 microscope (Olympus UK, Southendon-Sea, UK). A counting grid was applied to each image and oligodendrocyte cell bodies within this grid were counted. A total area of $0.25mm^2$ was measured within each animal. Sections single labelled for CC1 were analysed. The specificity of CC1 as a marker of oligodendrocytes was characterised by double labelling sections from the young 6-month cohort with astrocytic marker glial fibrillary acidic protein (GFAP). Double labelling was carried out by labelling CC1 brown by DAB and GFAP violet by peroxidise chromogen VIP (Vector, VIP). Astrocytes could be clearly identified by the presence of cell bodies. Counting was carried out using a counting grid at x200 magnification on a Leica DMR microscope within the same area of $0.25mm^2$. Astrocytes labelled with CC1 positive cells bodies were counted and found to represent less than 1% of the population of cells, validating CC1 as a specific marker of oligodendrocytes (fig 2.3).

2.2.6.2. Quantification of axonal integrity

Amyloid precursor protein (APP) was used as a marker of axonal integrity and assessed in the corpus callosum, fimbria, internal capsule and subcortical thalamic region. Sections were screened for the presence or absence of intense APP aggregation characterised by axonal bulbs (McKenzie et al., 1996). Quantification of

axonal integrity was analysed using a light microscope with sections screened for the presence or absence of axonal bulbs. Localisation of APP aggregation within the axon was identified by a haemotoxylin counterstain.

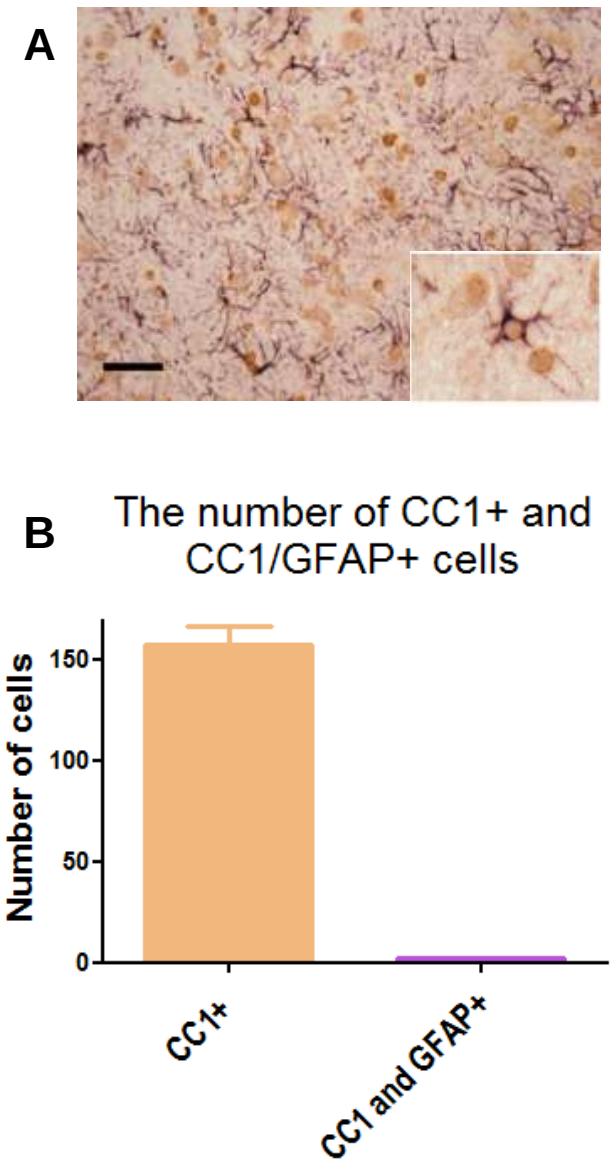

Figure 2.3: Specificity of CC1 as a marker of oligodendrocytes

Investigation of the specificity of CC1 (brown) as a marker of oligodendrocytes was carried out by double labelling sections with astrocytic marker GFAP (purple). (A) Representative image of CC1/GFAP double labelling with insert showing colocalisation. (B) Quantification of the number of CC1/GFAP positive staining compared to CC1 alone was found to present less than 1% of the population of the total cell count.

2.3. Tissue preparation for biochemistry

Animals selected for biochemical and microarray analysis were saline perfused and the brains snap frozen. Prior to freezing the brain was sectioned using a rat matrix (Fisher scientific, UK). A slice including the region corresponding to -3.14mm to -5.15mm from bregma (Paxinos and Watson 1998) was used for microarray analysis, with the remaining hemisphere processed for biochemical vessel enriched analysis.

2.3.1. Vessel enriched homogenate

Tissue was processed as a vessel enriched homogenate by density centrifugation using Ficoll™ (Sigma-Aldrich, Missouri, US). Gentle homogenisation of the tissue was carried out using ice cold PBS with a loose fit Dounce homogeniser. Cellular debris were removed by low speed centrifugation in an Eppendorf temperature controlled centrifuge (Eppendorf UK Ltd., Stevenage UK) at 250g for 15 minutes and the supernatant discarded. 17.5% Ficoll™ (Sigma-Aldrich, Missouri, US) solution in distilled water was added to the pellet, centrifuged for 25 minutes at high speed 3200g and a temperature of 4˚C. The supernatant was removed and the pellet washed with 1% BSA in PBS. Following centrifugation the supernatant was removed and the pellet was washed with PBS. This solution was centrifuged at 2000g for 20 minutes discarding the supernatant; the pellet was snap-frozen and stored at -80˚C.

2.3.1.1. Homogenisation of vessel enriched fraction for biochemistry

The vessel enriched fraction was homogenised for biochemical analysis in buffer containing 250mM sucrose, 20mM Tris Base, 1mM

ethylenediaminetetraacetic acid (EDTA) and 1mM ethylene glycol tetraacetic acid (EGTA) in distilled water (tissue homogenisation buffer). Protease and phospatase inhibitors (Merck, UK) were added to the tissue homogenisation buffer at concentrations of 1:100 and 1:50 respectively. Samples were homogenised in 150µl of buffer containing inhibitors using an electric homogeniser (Stuart, Bibby Scientific Ltd., Staffordshire, UK) at constant speed for 15 seconds. Samples were spun for 5 minutes at 3000rpm in a sigma 1-13 benchtop centrifuge (Sci Quip Ltd., Schrewsberry, UK), the supernatant removed and samples stored at -80˚C.

2.3.1.2. Determination of the protein concentration of samples for biochemistry

A bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Cramlington, UK) was used to determine the protein concentration within samples. Standards were made as per manufacturer's instructions and then pipette into appropriate wells of the 96 well plates. Samples were diluted in 1:10 in homogenisation buffer minus inhibitors onto a 96 well plate. The working reagent containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide, mixed in a 50:1 dilution with 4% cupric sulphate solution and added to each well, then incubated at 37˚C for 30 minutes. The plate was allowed to cool for 15 minutes and absorbance was read at 562nm on a LT-4000 MS microplate reader (Labtech, East Sussex, UK). Values generated from protein standards were used to plot a standard curve from which the protein concentration of each sample was calculated.

2.3.2. Western blotting

Samples were made up by concentrations of 1µg/µL with 0.25µL of NuPAGE® loading buffer (Invitrogen, Life technologies, New York, USA) and 0.1µL of NuPAGE® reducing agent (Invitrogen, Life technologies, New York, USA) per µL and the same buffer which was used for tissue homogenisation. Samples were incubated in a water bath at 70˚C for 10 minutes to denature proteins then loaded with a molecular weight marker (Li-Cor, Cambridge, UK) on precast 4-12% Bis/Tris gels (Invitrogen, Paisley, UK). Samples were loaded between two gels with a control sample repeated to check for consistency. Protein separation was carried out by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with 2-(N-morpholino) ethanesulfonic acid (MES) running buffer (Invitrogen, Paisley, UK). Samples were run through the gel at 100V, until bands reached the middle of the gel where the voltage was increased to 150V, until samples ran to the bottom of the gel.

Proteins were transferred on to polyvinylidene fluoride (PVDF) membrane (GE Healthcare, Little Chalfont, UK) in a XCell II Blot Module (Invitrogen, Paisley, UK) at 30V for 1.5 hours. To access the quality of transfer the membrane was stained with Ponceau S stain, which allowed for visualisation of banding. After assessment, if successful, membranes were washed with PBS then incubated in blocking solution of 1:1 odyssey blocking buffer (LI-COR, US) and PBS for 1 hour. Blocking was carried out in light proof containers, which were continually agitated. Primary antibody solution was made in blocking buffer, including α-Tubulin (Abcam, Cambridge, UK) at concentration of 1:20 000 and 0.1% tween-20, incubated at 4˚C overnight.

The following day primary antibody solution was drained off and membranes were washed in PBS 0.1% Tween; 6 times for 5 minutes each wash. Membranes were then incubated for 45 minutes in the appropriate secondary antibodies bound with infra-red dye (Li-Cor, Cambridge, UK) at concentration of 1/3000 mixed with blocking buffer containing 0.1% tween-20 and 0.01% sodium dodecyl sulphate. After secondary incubation membranes were washed 6 times in PBS 0.1% Tween for 5 minutes with a final 5 minute wash in PBS only. Membranes were dried between two pieces of filter paper and stored in light proof packaging. To identify fluorescent banding, membranes were scanned using an Odyssey infrared scanner (Li-cor, Cambridge, UK). Details of all antibodies used can be found in table 2.3.

2.3.2.1. Determination of protein levels by Western blotting

After membranes were dried and scanned the protein levels of bands could be calculated. The membranes were analysed using odyssey software (version 3.0; Li-Cor, Cambridge, UK), which allowed for the intensity of each band to be calculated. The intensity of each protein band of interest was expressed as ratio of the intensity of α-Tubulin band. All gels were run in duplicate to replicate findings and averaged for analysis.

Prior to running the vessel enriched samples, characterisation of vessel homogenate were undertaken. One sample was run in comparison to a whole brain homogenate to examine protein content of vascular markers. As can be seen in figure 2.4, the vessel enriched sampled allowed detection of vascular markers, whereas the whole brain sample did not.

Primary antibody	Supplier	Dilution	Secondary antibody	Amount of protein loaded
α-Smooth muscle actin	Millipore, Watford, UK	1:1000	Anti mouse	15 µg
PECAM	Abcam, Cambridge, UK	1:50	Anti rabbit	15 µg
Claudin-5	Invitrogen, Califonia, USA	1:1000	Anti mouse	15 µg
ZO-1	Invitrogen, Califonia, USA	1:500	Anti rabbit	15 µg
α-Tubulin	Abcam, Cambridge, UK	1:20000	Anti mouse	15 µg

Table 2.3: Details of antibodies used in Western blotting assessment
Details the antibodies use for western blotting protein assessment in vessel enriched homogenate including manufacturer, dilution and secondary antibody used.

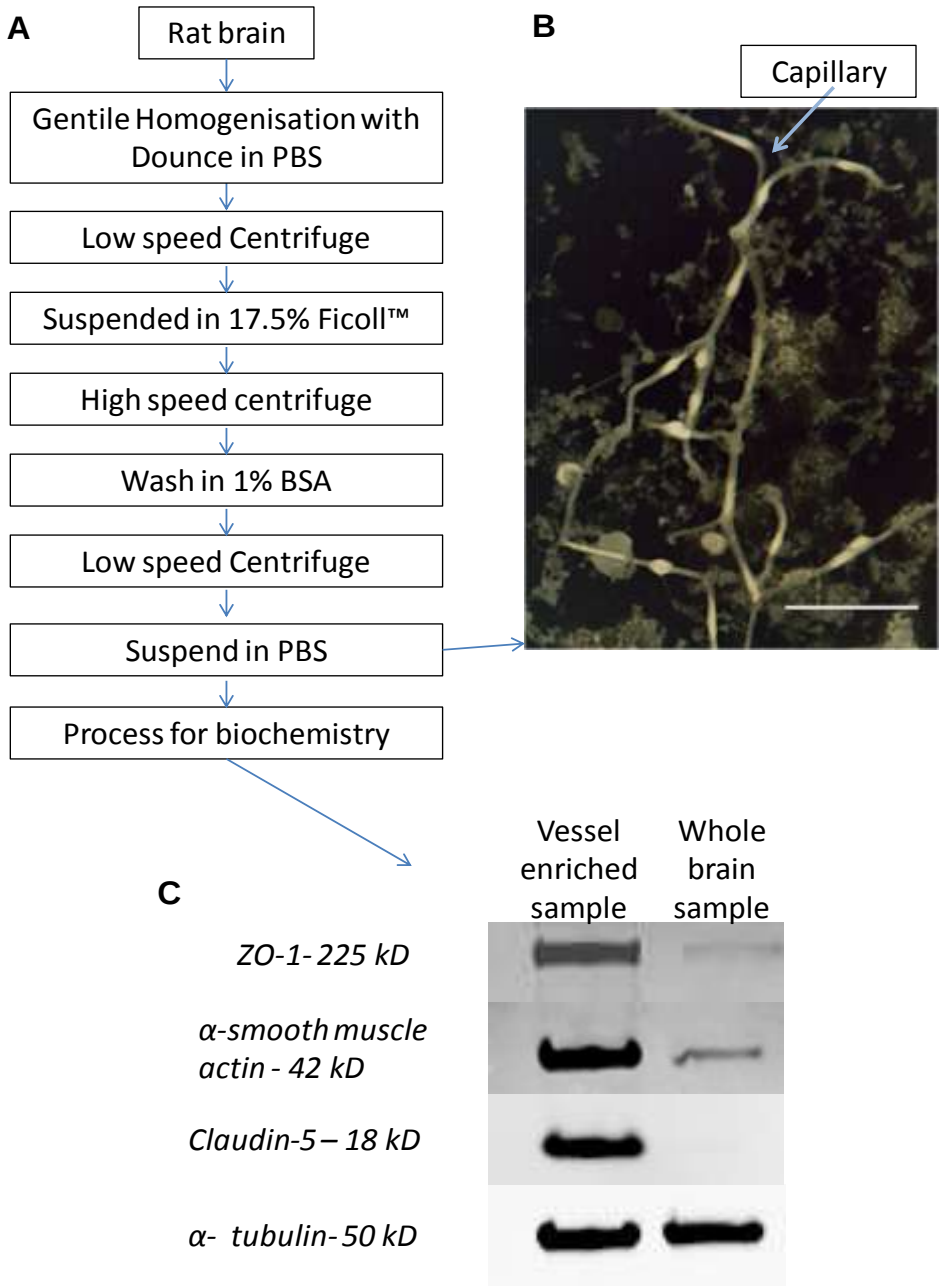

Figure 2.4: Characterisation of vessel enriched homogenate

Representative image of the methodology and the cellular components within the vessel enriched homogenate. (A) Flow chart of the gradient centrifugation steps required to extract vessels. (B) Representative visualisation of vessel enriched homogenate on a phase contrast microscope. The image demonstrates a sample, which is enriched in vessels and that the overall structural integrity of the vasculature is intact. (C) Characterisation western blots. Within equal protein levels the vessel enriched homogenate allows identification of vascular specific proteins which are not detected in the whole brain homogenate. α-Tubulin was used as a loading control which is present at equal levels between the two fractions.

2.3.3. Microarray

2.3.3.1. RNA extraction

Tissue processed for microarray was taken from a subcortical dissection of the same tissue used for biochemical analysis. Firstly the tissue slice was allowed to thaw and the subcortex was carefully dissected with the remaining tissue disposed of. Ribonucleic acid (RNA) was extracted using Trizol™ protocol according to manufacturer's instructions (Invitrogen, Paisley, UK). Succinctly, tissue was homogenised in 1ml/100mg of Trizol™ using lysing matrix tubes in a MP Biomedical FastPrep Homogeniser (Fisher scientific, Leicestershire, UK). Phase separation was carried out by addition of 1-bromo-3-chloro propane and samples were centrifuged at 12 000g for 15 minutes, with the upper phase transferred into a new tube. The upper phase was used for RNA precipitation with the addition of linear polyacrylamide and a further high speed centrifugation. RNA formed a gel-like pellet, the supernatant was discarded and RNA was washed with 75% Ethanol followed by distilled water. After washing the pellet was allowed to air dry until the circumference of the pellet turned white and then re-suspended in distilled water for storage at -80˚C.

2.3.3.2. Determination of RNA concentration and integrity

RNA concentrations were determined using a NanoDrop 3300 Fluorospectrometer (Thermo scientific, USA) and RNA integrity analysed using Agilent 2100 Bioanalyser (Agilent Technologies, Inc., Colorado, US), separating nucleic acid fragments by molecular weight and laser induced fluorescence. An Agilent RNA 6000 Nano kit (Agilent Technologies, Inc., Colorado, US) was used

with samples pipette onto the Nanochip, containing a sieving polymer fluorescent dye and external standards ladder. The Bioanalyser then electrophoretically separated the RNA within each sample by size and the intercalated fluorescent dye is detected at different speeds within different bands. The RNA ladder contains 6 fragments, from 0.2 to 6km, at a concentration of 150ng/µl and the Bioanalyser software compares the unknown samples to the ladder fragment to determine the concentration and ribosomal peaks. Data from the Bioanalyser was represented by the electrophoresis, showing band separation of RNA fragments by size and the integrity of RNA was evaluated by the electropherogram trace and RNA integrity number (RIN). The electropherogram of intact RNA will demonstrate two distinct ribosomal peaks corresponding to 18S and 28S RNA with no other peaks between the 18S and 5S peak (Fig 2.5). RNA integrity was also calculated by the RIN number, based on a software algorithm on a scale of 1-10, with 1 representing completely degraded RNA, whereas 10 is intact. More samples were processed than were needed, with all samples having a high RIN number thus, samples used for microarray were chosen at random.

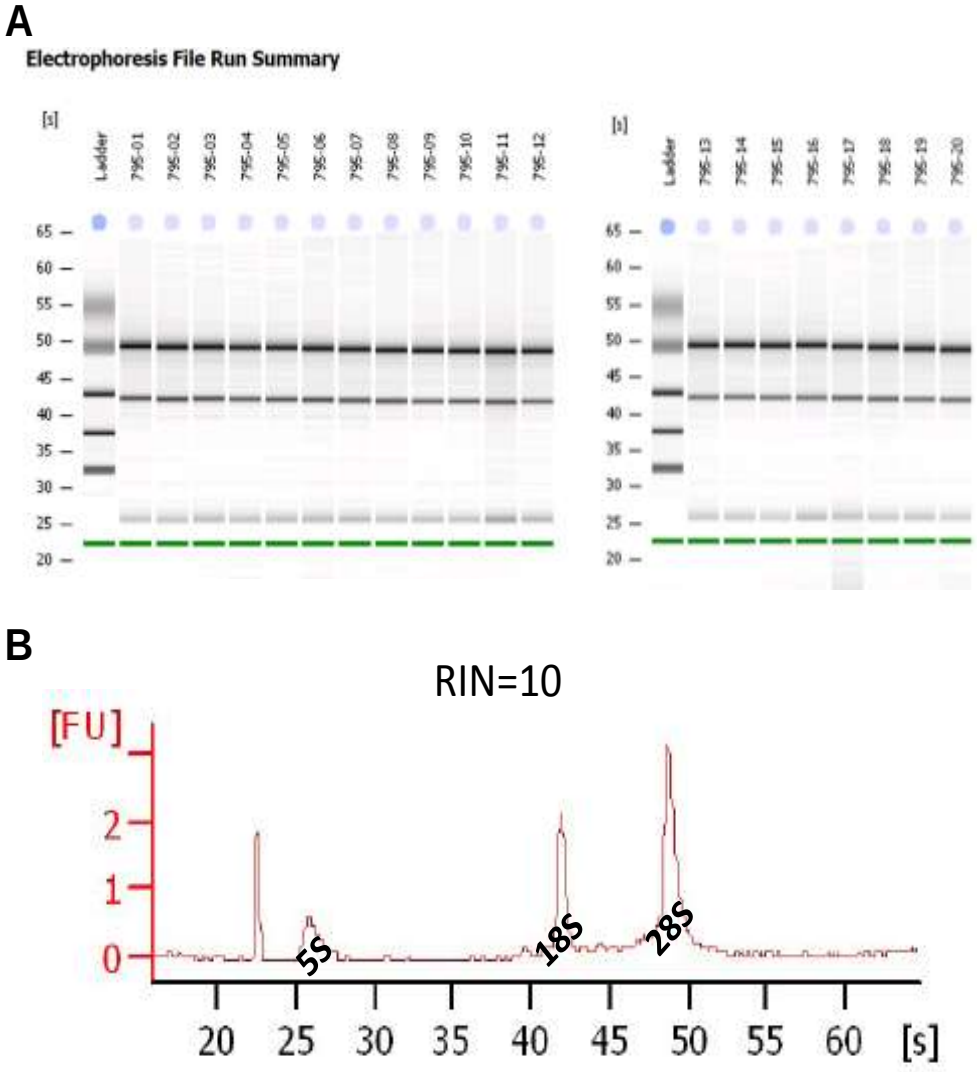

Figure 2.5: Integrity of RNA

Representative image of RNA integrity analysis. (A) Electrophoresis of RNA samples provided evidence of two distinct bands in each sample corresponding to 18s and 28s RNA. (B) Representative electropherograms of study RNA concentration showing two distinct 18s and 28s peaks with no degradation between the two or between the early 5s peak. RIN number is representative of the integrity of RNA within the sample on a scale of 1 to 10, fully degraded to fully intact respectively. All RIN numbers within study samples had a RIN number of ≥ 9.

2.3.3.3. Microarray gene expression profiling

Procedures such as cDNA preparation, microarray assay and analysis were carried out with the assistance of Alison Downing (Ark Genomics, Roslin Institute, UK). Firstly sense strand cDNA preparation from RNA samples was generated and labelled using Ambion WT expression kit (Affymetrix, US) and Affymetrix GeneChip WT terminal labelling kit array (Affymetrix, US), as per manufacturer's instructions. The gene expression console, GeneTitan® was used to hybridise cDNA from each sample onto a Genechip strip rat gene 1.1 array (Affymetrix, US), >27 000 genes, followed by washes and scanning of the intensity of each probe. Signal intensities were screened for outliers by plotting the log probe cell intensity for each sample (Figure 2.6a) and data normalised by robust multichip average (RMA) analysis method, using the Expression console™ (Affymetric, US). Raw data was used to run principal component analysis (PCA) and the sum of squares analysis using Genomics suite (Partek® Inc, US) to visualised sources of variation (figure 2.6b). Log2 transformed normalization signal intensities of annotated transcripts were analysed by one way analysis of variance (ANOVA), with the source of variation - perfusion date attributed as a factor (Partek® Inc, US). An overall significance was set at $p<0.005$, but, the high variability and low level of differentially expressed signal intensities within the data set did not allow any differentially expressed genes to pass false discovery rate (FDR) correction. However, due to the previous findings in this study it was anticipated that differentially expressed genes would be relatively subtle, but it was not appreciated after the high quality of RNA yielded that there would be a high level of variability.

Pathway analysis was used to allow the mapping of biological functional networks, carried out using IPA software (Ingenuity systems, US) with differentially expressed genes of significance of $p<0.01$. The 4 most significantly altered networks were ranked by a score value calculated by negative log of the *p*-value.

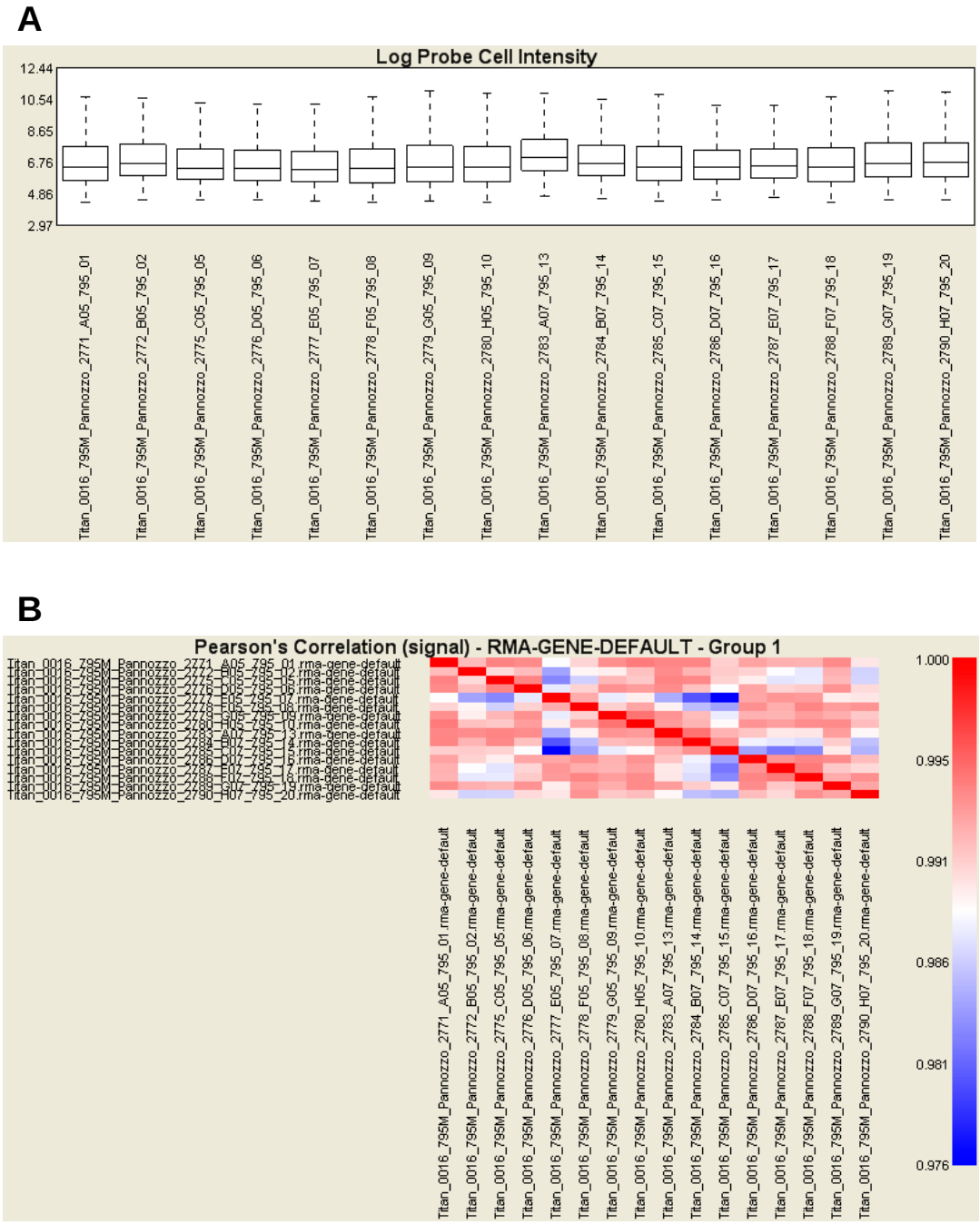

Figure 2.6a: Microarray intensity signal normalisation

(A) Average log probe cell intensity for each animal prior to normalisation indicates no outliers. (B) Pearson's correlation of average intensity values for each animal provides evidence of a positive correlation and no outliers

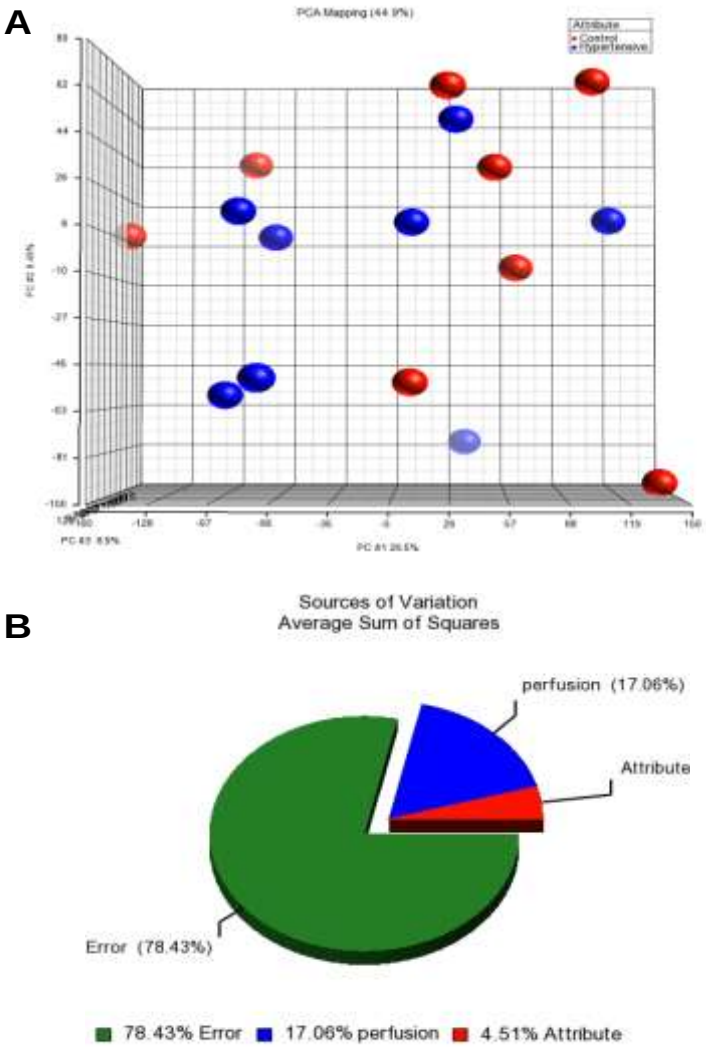

Figure 2.6b: sources of variation within microarray intensity signals
Representative image of the variance within microarray log intensity signals. (A) Principle component analysis (PCA) plot representative of sources of variance within log signal intensities for each subject. Normotensive controls are show in red and hypertensive animals are shown in blue. The PCA plot highlights outliers within the study and provides evidence of clustering of subjects independent of treatment. (B) Pie chart representing the variation within the study. A high source of variation is labelled as error and of unknown cause. The other sources of variance observed within this study were perfusion date and attribute (treatment). The high level of unattributed variance compared to the attributed variance did not allow any individual genes to pass the FDR correction.

2.4. Behavioural testing

Prior to behavioural testing animals were habituated to handling due to frequent blood pressure recordings. Behavioural testing was carried out in pairs of experimenters blinded to treatment groups with the order of cognitive testing counterbalanced between treatment groups, allowing one individual to work the recording software and the other to place each animal in the maze. Water maze testing was run by myself, Dr Chen and Miss Smith and all the data was analysed by myself.

2.4.1. Morris water maze

Water maze protocols were conducted in a circular pool (200cm diameter, Figure 2.7), filled with 25 ± 1°C opaque water (by addition of 400ml latex liquid). The water maze room had many extra-maze 2D and 3D visual cues, encompassing brightly coloured geometric images. During cognitive testing the experimenter and awaiting rats were hidden behind white curtains to prevent those becoming extra interchangeable cues. The maze activity of each rat was monitored via CCTV camera mounted above the pool. This allowed parameters such as the latency to find the platform to be tracked and monitored using Water Maze tracking software (ActiMetrics software version 2.6).

2.4.1.1. Cue task

Prior to water maze testing animals were trained with a cue task version of the water maze in which any gross motor or visual impairment could be observed. In the cue task the target platform had a diameter of 20cm and was visualised by a 20cm high cue. Extramaze clues were precluded from this task by a white curtain

around the maze. The platform was located in a different quadrant for each trial on a given day. Animals were given 3 trials per day for 4 consecutive days. During a trial each animal was placed in the water maze facing the wall of the pool and allowed 90 seconds of swimming to locate the platform. The animals starting position was counterbalanced between quadrant and platform location, so not to repeat a starting position on any day.

2.4.1.2. Spatial reference learning and memory

Spatial reference memory testing occurred 3 days after the Cue task. The Atlantis platform (13cm in diameter) was submerged approximately 1 cm below the surface of opaque water, uncued and not visible to animals. Animals were allowed 4 trials per day for 5 days to locate the hidden platform. The platform location was constant throughout the trial but the animals starting location, counter balanced to platform location across the group and varied each trial. 10 minutes after the final spatial reference learning task animals were given a probe trial in which the platform is fully submerged in the location of the spatial reference trial and rises after 1 minute of searching. 24 hours later the probe trial is repeated with no platform present.

2.4.1.3. Delayed matching to place protocol

Delayed matching to place protocol was carried out 3 days after the spatial reference learning task based on protocol devised by Steel and Morris (Steele and Morris, 1999). The 13cm platform was relocated each day, with animals given a longer 20 minute inter-trial interval between trails 1 and 2 and a shorter 15 second inter-trial interval between trials 2, 3, and 4. The delayed matching to place task was

run for 4 consecutive days with the starting location of the animal varied and counter balanced to platform location each day.

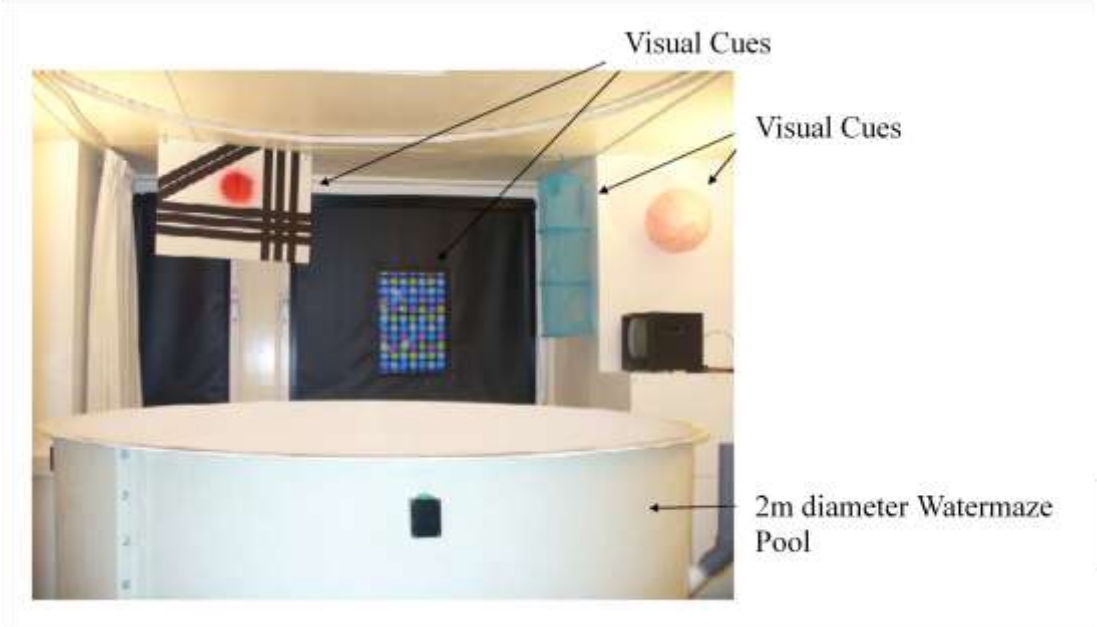

Figure 2.7: The Morris Watermaze

Image of the Morris Watermaze showing a selection of both 2D and 3D visual cues and the fibre glass pool.

2.5. Statistics

2.5.1. Blood pressure

Statistical comparisons of systolic blood pressure between normotensive and hypertensive animals for each individual cohort separately (young 4 month, young 6 month and age 4 month) was firstly analysed using repeated measures ANOVA with significance set at $p<0.05$, to allow an understanding of the significance throughout the duration of the study. Following posthoc analysis was carried out using student t-test at specific time points of week 0, 5 and end-stage to confirm significance throughout with significance set at $p<0.01$.

2.5.2. Pathology

2.5.2.1. Vascular pathology

The majority of immunohistochemical data was presented as mean ± standard error of the mean, apart from vessel width measurements presented as cumulative frequency and the number of eNOS positive vessels presented as percentage of controls. All the relative optical density measurements (ROD) and count data were examined by Student's t-test. Density measurements analysed by percentage area stained, were examined by Mann Whitney U test. Vascular width measurements were examined by Kolmogorow - smirnov test. Microglial vascular association data were examined by Fishers exact test. The significance for immunohistochemical data was set at $p<0.02$. Throughout this thesis each cohort is analysed separately and comparisons were not made between cohorts. Unfortunately as each was ran distinct to each other, there were several variable factors such as housing during the study, tissue processing, immunohistochemistry and pathological analysis. As several

statistical assessment were made per cohort the significance was set more stringently to $p<0.02$, in an aid to account for false positives.

2.5.2.2. White matter pathology

The majority of data was presented as mean ± standard error of the mean, apart from MBP intensity and microglia count data, presented as percentage of controls. Data for MBP intensity values, CC1 positive cell counts and number of microglia were analysed using Student's t-test. APP data was analysed by Fisher's exact test. The significance was set at $p<0.02$, as described above and each cohort was analysed separately.

2.5.2.3. Western blotting data

The integrated intensity measurements taken from western blotting experiments were expressed in relation to α-tubulin, data presented as mean ± standard error of the mean and examined by Student's t-test. Significance was set at $p<0.05$.

2.5.2.4. Microarray Gene expression data

The normalized log2 signal intensities were analysed using ANOVA with perfusion date as a factor and significance set at $p<0.005$. A separate analyse was carried out with a significance of $p<0.01$ for pathway analysis.

2.5.2.5. Behavioural assessments

Behavioural data was presented as mean ± standard error of the mean, analysed using two-way ANOVA with repeated measures and significance set at $p<0.05$.

3. The effect of inducible hypertension on the structural integrity of the cerebrovasculature in young and aged rats

3.1. Introduction

The structural integrity of the cerebrovasculature is crucial for normal brain function. Hypertension has been associated with cerebrovascular remodelling in humans, commonly located within the subcortical structures of the brain and thought to underlie gross structural and functional alterations (Fisher, 1969, 1971; Lammie, 2002). Data from the SHR and SHRSP models of hypertension have also found evidence of vascular remodelling (Baumbach and Heistad, 1988; Baumbach and Hajdu, 1993; Knox et al., 1980; Sabbatini et al., 2001; Tagami et al., 1987). The present study sought to investigate the effect of sustained hypertension on subcortical cerebrovascular structure in the young and aged Cyp1a1 Ren2 transgenic rats.

3.1.2. Hypothesis

Hypertension will lead to vascular alterations within the subcortex of the young and aged brain consisting of remodelling of the basement membrane, loss of BBB integrity and altered endothelial signalling.

3.1.3. Aims

The study in this chapter aimed to investigate the effect of hypertension on the structural integrity of the vasculature in the young and aged brain of the Cyp1a1 Ren2 rat model. A detailed examination of vascular width and density, endothelial signalling, endothelial blood-brain barrier integrity and the inflammatory response was carried out.

3.2. Methods

3.2.1. Animals

Experiments were conducted on Cyp1a1 Ren2 transgenic rats described in chapter 2.1, separated into young and aged cohorts. Rats were either attributed to the normotensive group, fed a control diet consisting of no I3C or the hypertensive group, fed a diet consisting of 0.15% I3C. The young cohort contained two groups, run for different durations (4 or 6 months). The young 4-month cohort consisted of normotensive (control diet, no I3C, n=12) and hypertensive (0.15% I3C, n=16) animals. The young 6-month cohort included normotensive (control diet, no I3C, n=21) and hypertensive (0.15% I3C, n=21) animals. At termination, the 6-month cohort was separated into those processed for immunohistochemistry; normotensive (control diet, no I3C, n=13) and hypertensive (0.15% I3C, n=13) and those processed for biochemistry; normotensive (control diet, no I3C, n=8) and hypertensive (0.15% I3C, n=8). The aged cohort was run for a duration of 4 months including; normotensive (control diet, no I3C, n=10), and hypertensive (0.15% I3C, n=12) animals. All animals from the young 4-month cohort survived well. However, 4 hypertensive animals from the young 6-month cohort and 2 animals (1 normotensive, 1 hypertensive) from the aged 4-month cohort were prematurely culled due to excessive weight loss.

3.2.2. Measurements of blood pressure

Throughout the study blood pressure measurements were carried out (described in chapter 2.1.3.2), using the tail-cuff plethysmography method. All rats were trained and habituated for a period of 2 weeks prior to dietary induction. During

blood pressure measurements the animals' general health was also recorded including their weight, gate and grooming.

3.2.3. Immunohistochemistry

Pathological assessments were carried out on paraffin embedded brain tissue sections (6μm) from young and aged cohorts (chapter 2.2). Sections encompassing the subcortical thalamic region of the brain (chapter 2.2.4) were stained to visualize components of the vasculature using markers for basement membrane; collagen IV (2A), smooth muscle cells; α-smooth muscle actin, endothelial tight junction; claudin-5 and ZO-1, endothelial; eNOS and microglial; Iba1. Full technical methodology and quantification of vascular markers used within this chapter are outlined in detail in chapter 2.2.

3.2.4. Global assessment of vascular protein levels

Investigation of global vascular protein levels were assessed in the young 6-month cohort, by the generation of a vessel enriched homogenate, from one hemisphere of the brain (excluding the slice used for microarray investigation), described in full in chapter 2.3. Protein levels were assessed using western blotting technique, performed to detect α-smooth muscle actin, PECAM, claudin-5 and ZO-1, with α-tubulin used as a loading control.

3.2.5. Statistical analysis

Statistical comparisons of systolic blood pressure between normotensive and hypertensive animals for each individual was firstly analysed using repeated measures ANOVA with significance set at $p<0.05$, to allow an understanding of the significance throughout the duration of the study. Followed by posthoc analysis was

carried out using student t-test at specific time points of week 0, 5 and end-stage, with significance set at $p<0.01$. Immunohistochemical and western data presented as mean ± standard error of the mean, examined by Student's t-test, apart from percentage area stained data, which was analysed by Mann Whitney U test and microglia association data, which was examined by Fishers exact test. The significance for immunohistochemical data was set at $p<0.02$ and $p<0.05$ for Western data. Throughout the chapter each cohort was analysed separately and comparisons between cohorts were not made.

3.3. Results

3.3.1. Indole-3-carbinol caused sustained increased blood pressure

3.3.1.1. Blood pressure in the young 4-month cohort

Throughout the duration of the study it was found that there was a significant difference in blood pressure over time ($F_{(8.98,\ 4769.38)} = 15.66$, $P<0.01$). Interestingly, there was a significant deference between blood pressure and treatment ($F_{(8.98,\ 5535.49)} = 18.16$, $P<0.01$) in control animals versus those fed a hypertensive diet.

The profile of blood pressure was also examined at specific time points throughout the study. At week 0, blood pressure for the young 4-month cohort was; 128 ± 4 mmHg for animals assigned to the control diet group versus 124 ± 2 mmHg for animals assigned to the hypertensive diet group ($|t|= 1.37$, df= 15, $p=0.19$; Figure 3.1A) and was found not to be significantly different. At week 5 post dietary induction, there were significant differences in systolic blood pressure between animals fed a control diet and animals fed a hypertensive diet in the young 4-month

cohort; blood pressure was 136 ± 3 mmHg for control diet group versus 176 ± 4 mmHg for hypertensive diet group (|t|= 3.10, df= 17, p<0.01; Figure 3.1A). End-stage blood pressure was significantly different between animals fed a control diet and animals fed a hypertensive diet in the young cohort with blood pressure of 135 ± 2 mmHg for animals fed a control diet versus 195 ± 2 mmHg for animals fed a hypertensive diet (|t|= 13.33, df= 14, p<0.01; Figure 3.1A).

3.3.1.2. Blood pressure in the young 6-month cohort

Throughout the duration of the study it was found that there was a significant difference in blood pressure over time ($F_{(6.04,\ 5295.14)}$ = 14.16, P<0.01). Interestingly, there was a significant difference between blood pressure and treatment ($F_{(6.04,\ 11456.55)}$ = 30.63 P<0.01) in control animals versus those fed a hypertensive diet. At week 0, blood pressure for the young 6-month cohort was; 146 ± 2 mmHg for animals assigned to the control diet group and 145 ± 3 mmHg for animals assigned to the hypertensive diet group (|t|= 0.16, df= 15, p=0.86; Figure 3.1B). At week 5 post dietary induction, there were significant differences in systolic blood pressure between animals fed a control diet and animals fed a hypertensive diet in the young 6-month cohort with blood pressure of; 147 ± 3 mmHg for animals fed a control diet and 160 ±2 mmHg for animals fed a hypertensive diet (|t|= 3.77, df= 19, p<0.01; Figure 3.1B). End stage blood pressure for the young 6-month cohort was 131 ± 2 mmHg for animals fed a normotensive diet and 185 ± 2 mmHg for animals fed a hypertensive diet (|t|= 10.90, df= 21, p<0.001; Figure 3.1B).

3.3.1.3. Blood pressure in the aged 4-month cohort

Throughout the duration of the study it was found that there was a significant difference in blood pressure over time ($F_{(7.14,\ 2700.54)} = 10.47$, $P<0.01$). Interestingly, there was a significant difference between blood pressure and treatment ($F_{(7.14,\ 1195.40)} = 4.63$ $P<0.01$) in control animals versus those fed a hypertensive diet. At week 0, blood pressure for the aged 4-month cohort was; 145 ± 4 mmHg for animals assigned to the control diet and 150 ± 2 mmHg for animals assigned to the hypertensive diet (|t|= 0.11, df= 15, p=0.91; Figure 3.1C). At week 5 post dietary induction, there were significant differences in systolic blood pressure between animals fed a control diet and animals fed a hypertensive diet and blood pressure was 128 ± 2 mmHg for animals fed a control diet and 158 ± 4 mmHg for animals fed a hypertensive diet (|t|= 9.06, df= 14, $p<0.001$; Figure 3.1C). At end stage, the aged 4-month cohort had blood pressure of 140 ± 2 mmHg for animals fed a control diet and 182 ± 4 mmHg for animals fed a hypertensive diet (|t|= 9.07, df= 14, $p<0.001$; Figure 3.1C).

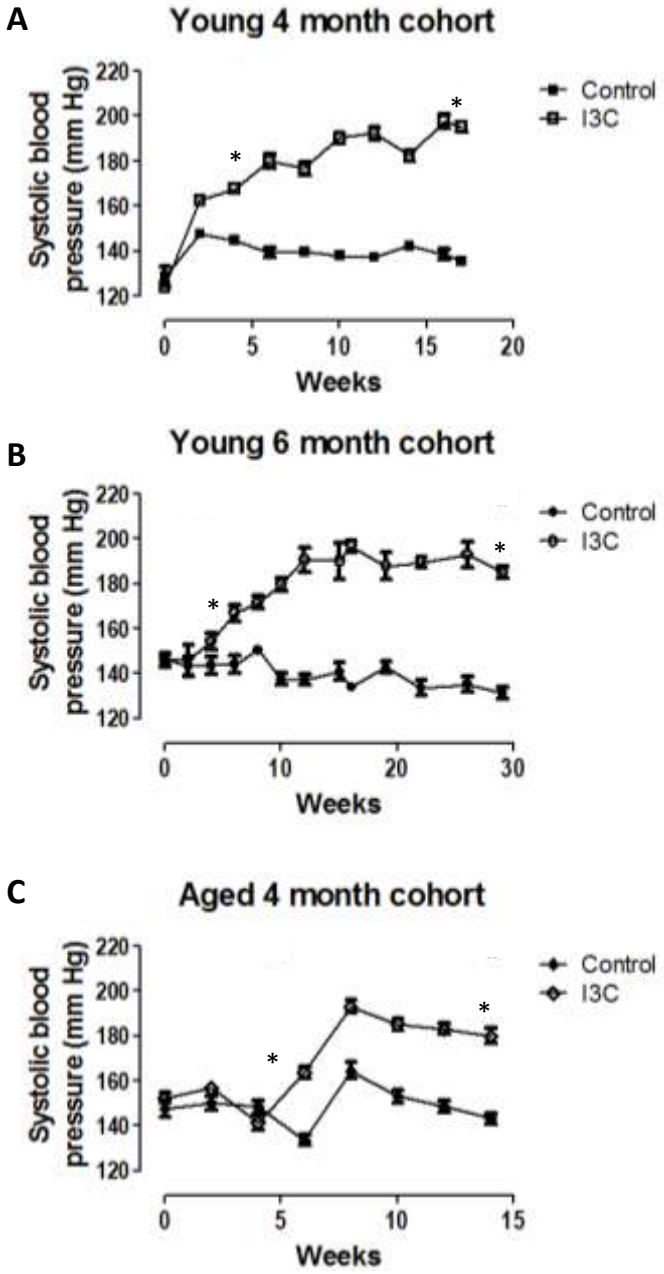

Figure 3.1: Systolic blood pressure

Systolic blood pressure increased with dietary addition of I3C and sustained over time. (A) Young 4-month cohort blood pressure was significantly increased at week 5 in animals fed I3C compared to animals fed a control diet. End-stage blood pressure was 135 ± 2mmHg for animals fed a control diet and 195 ± 2mmHg for animals fed an I3C containing diet. (B) Young 6-month cohort animals fed an I3C containing diet had significantly increased blood pressure at week 5 when compared to those fed a control diet, end-stage blood pressure was 131 ± 2mmHg control versus 185 ± 2mmHg I3C diet. (C) I3C fed animals from the aged 4-month cohort had significantly increased blood pressure when compared to those fed a control diet, at week 5 with end-stage blood pressure values of 140 ± 2mmHg control diet versus 182 ± 2mmHg for I3C diet. Graphs show mean ± SEM. * = $p < 0.01$ hypertensive vs.control.

3.3.2. Hypertension induced alterations to vascular structure

Collagen IV (specific for Collagen IV 2A subunit), a marker of basement membranes, was used as a general indicator of vascular integrity. Collagen IV clearly outlined the structure of all vessels in both cross sectional and longitudinal orientations, allowing for the quantification of vascular density, width and number of vessels (Figure 3.2). For the analysis of vessel width, only the vessels in a longitudinal plane were quantified. In the young 4-month cohort, there was no significant difference in the density (p=0.47, Figure 3.2J, Table 3.1), vessel width (D= 0.09, p=0.13, Figure 3.2G, Table 3.1 Appendix A.1) or number of vessels ($|t|$= 0.64, df=28, p=0.53, Table 3.1, Appendix A.2) in hypertensive animals when compared to normotensive. Figure 3 presents the data for each cohort in frequency distribution with the raw vessel width presented in Appendix A.1.

However, in the young 6-month cohort vascular structure was altered in the hypertensive compared to normotensive rats. Vessels of the hypertensive animals displayed a string-like appearance (Figure 3.2E, Table 3.2), with a significant reduction in vessel width (D= 0.15, p<0.01, Figure 3.2H, Table 3.2, Appendix A.1) compared to normotensive animals. These alterations were in absence of a significant difference in vascular density (p=0.25, Figure 3.2K, Table 3.2) or the number of vessels ($|t|$= 1.29, df=21, p=0.21, Table 3.2, Appendix A.2), in hypertensive animals when compared to normotensive. Figure 3 presents the data for each cohort in frequency distribution and raw vessels width measurements plotted in Appendix A.1.

In the aged 4-month cohort, there was a significant increase in both the density (p=0.01, Figure 3.2L, Table 3.3) and number of vessels (|t|= 3.10, df=18, p=0.01, Table 3.3, Appendix B.2) in hypertensive animals when compared to normotensive, but there was no evidence of any difference in vascular width (D= 0.07, p=0.33, Figure 3.2I, Table 3.3, Appendix A.1). The vasculature of the aged hypertensive animals did not exhibit the string like appearance, as seen in the young 6-month cohort but they appeared denser. Figure 3 presents the data for each cohort in frequency distribution, raw vessel width plotted in Appendix A.1.

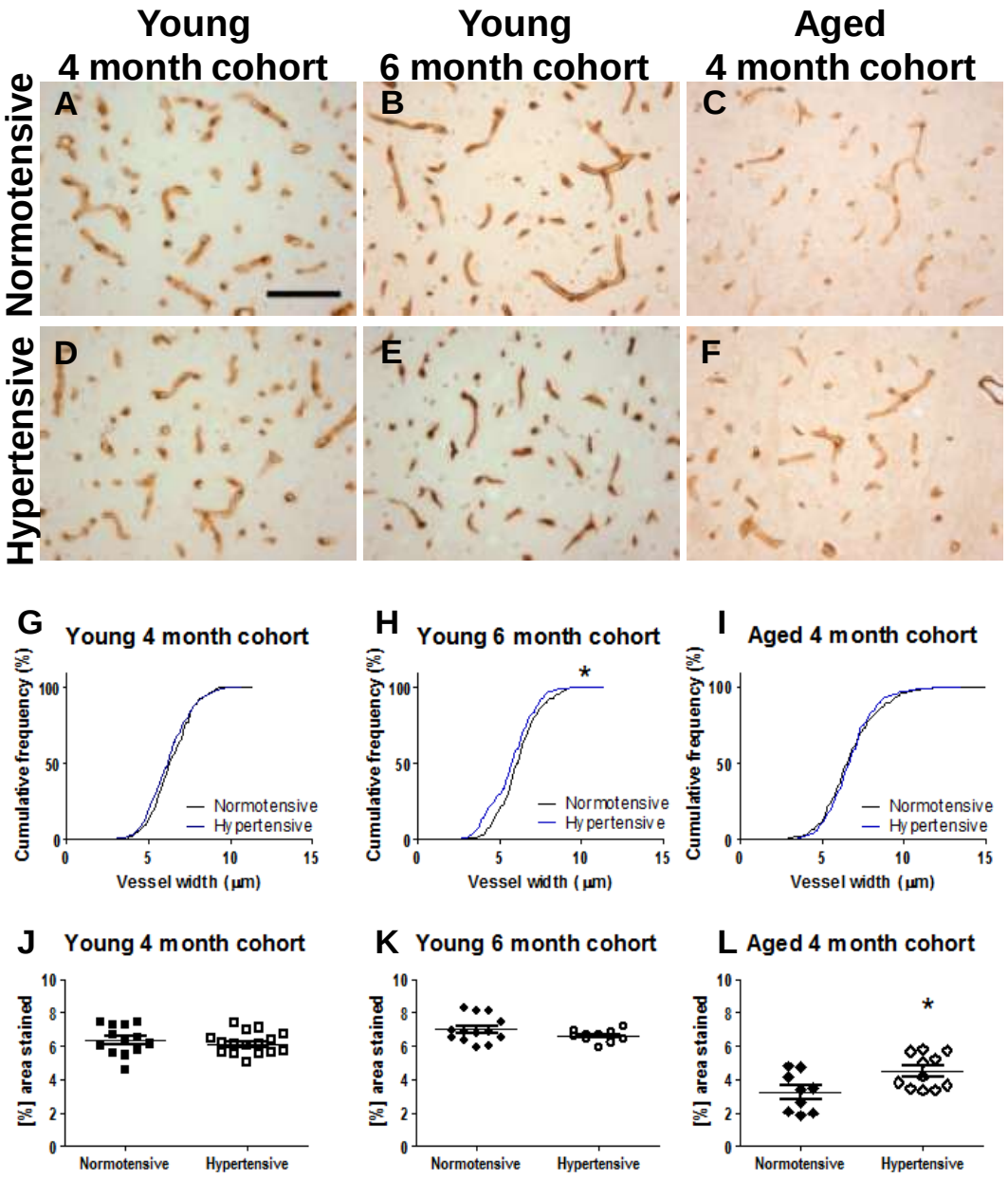

Figure 3.2: Assessment of cerebrovascular structure using Collagen IV staining

Representative images of cerebrovascular structure using antibody specific for Collagen IV (A2), in normotensive (A, B, C) and hypertension (D, E, F) animals from each cohort. In the young 4-month cohort there was no change in vascular width (G) or density (J) in the hypertensive animals (D) when compared to normotensive (A). In the young 6-month cohort hypertensive animals displayed thinner string like vessels (E), throughout a range of vessel sizes with an overall significant decreased in vessel width (H) represented by *, but not density (K), when compared to normotensive animals (B). The aged 4-month cohort had increased density of vessels in hypertensive (F,L) when compared to normotensive animals (C), with no alterations in vessel width (I). Scale bar = 100µm. Graphs show mean ± SEM. Significance *=p<0.02 hypertensive vs normotensive.

3.3.3. Hypertension does not induce overt structural alterations to the large arteries and arterioles

Smooth muscle actin was used as a general indicator of larger artery and arteriole integrity, as small arterioles and capillaries have minimal or no smooth muscle actin (Drake and Iadecola, 2007). This was shown in the profile of staining, with immunopositive vessels mainly large in size, in comparison to the array of vessels stained with collagen IV. In the young 4-month cohort, no significant difference in the density of smooth muscle actin was found between hypertensive and normotensive animals (p=0.26, Figure 3.3G, Table 3.1). Similarly, there was no significant difference in the density of staining of smooth muscle actin between hypertensive and normotensive animals from the young 6-month (p=0.26, Figure 3.3H, Table 3.2) and the aged 4-month (p=0.03, Figure 3.3I, Table 3.3) cohort. This is indicative that hypertension in this model primarily affects the smaller arterioles and capillaries, due to differences found within the basement membrane.

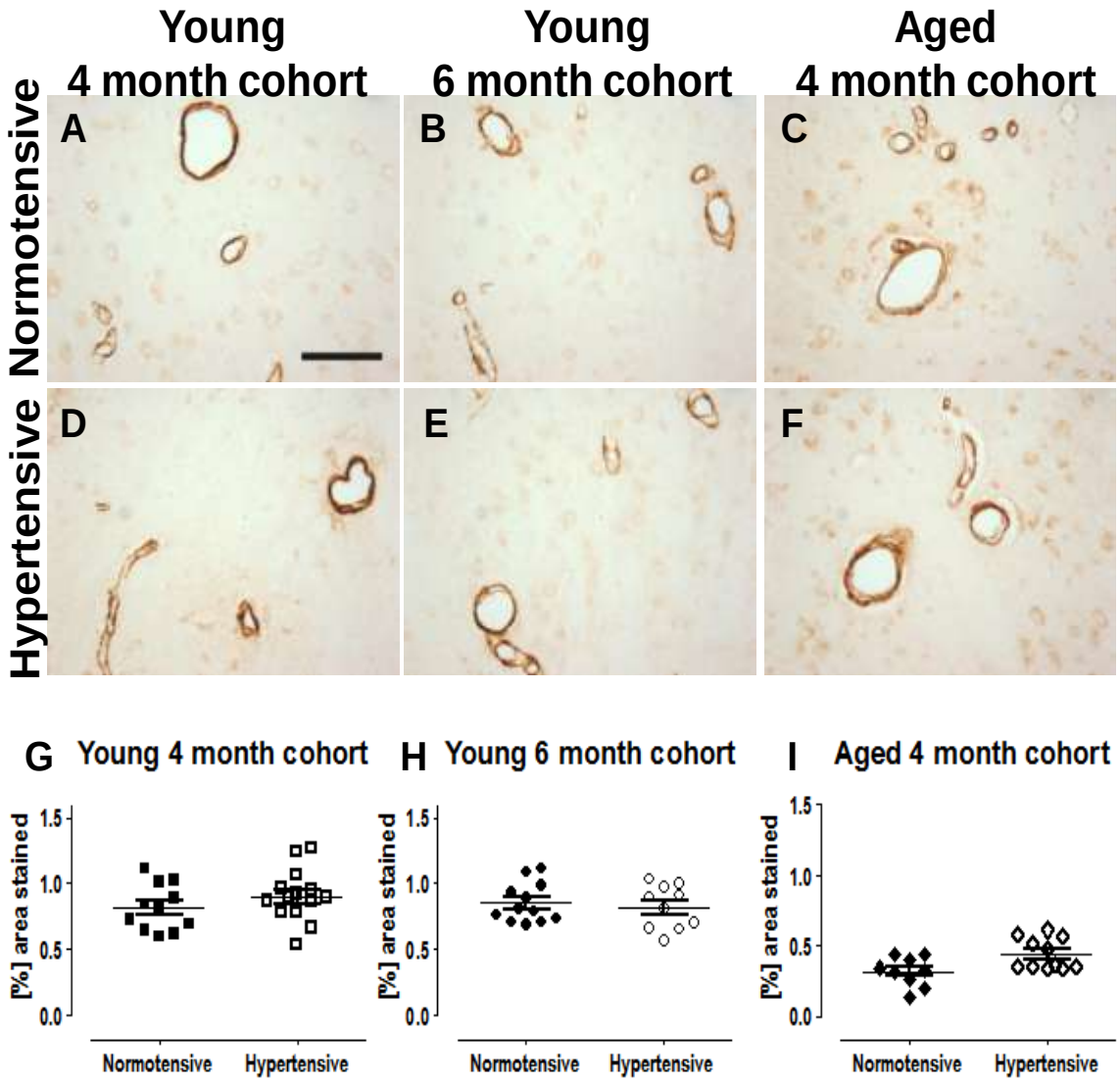

Figure 3.3: Structural assessment of large arteries and arterioles
Representative images of the structural integrity of large arteries and arterioles using an antibody specific for α-smooth muscle actin in normotensive (A, B, C) and hypertensive (D, E, F) animals. Overall, there was no significant difference in the density of vascular smooth muscle actin between normotensive (A, B, C) and hypertensive (D, E, F) animals from the young 4-month cohort (G), young 6-month cohort (H) or the aged 4-month cohort (I). Scale bar = 50µm. Graphs show mean ± SEM.

Vascular data for the Young 4-month cohort							
Collagen IV						Smooth muscle actin	
Vessel width (µm)		% area stained		Number of vessels		% area stained	
Normotensive	Hypertensive	Normotensive	Hypertensive	Normotensive	Hypertensive	Normotensive	Hypertensive
5.98	5.46	6.49	6.13	22.20	24.86	0.46	0.40
6.46	5.11	5.57	4.97	19.50	15.60	0.42	0.37
6.41	6.82	6.47	6.03	23.60	27.60	0.41	0.39
6.96	6.02	6.15	6.32	23.60	21.40	0.39	0.40
6.38	6.72	4.58	6.15	23.00	24.00	0.37	0.48
6.72	6.60	5.99	5.58	28.00	24.20	0.37	0.41
6.68	6.97	5.47	5.59	21.40	26.20	0.42	0.36
6.74	7.25	7.28	6.93	23.50	24.40	0.37	0.39
5.60	5.86	7.44	5.54	16.33	28.20	0.41	0.40
6.53	6.52	7.43	7.07	20.00	25.00	0.41	0.39
6.43	5.82	6.67	5.71	25.60	25.40	0.37	0.37
6.40	5.69	5.73	5.53	26.80	18.40	0.40	0.37
	6.52		6.46		23.00		0.43
	6.45		7.33		26.40		0.38
	6.64		6.11		18.75		0.34
	6.53		6.65		26.75		0.35

Table 3.1: Vascular data values for the young 4-month cohort

The above data table shows raw data values analysed for Collagen IV measurements; vessel width, % area stained and number of vessels and Smooth muscle actin analysis; % area stained.

Vascular data for the Young 6-month cohort							
Collagen IV						Smooth muscle actin	
Vessel width (µm)		% area stained		Number of vessels		% area stained	
Normotensive	Hypertensive	Normotensive	Normotensive	Normotensive	Hypertensive	Normotensive	Hypertensive
5.55	5.31	8.16	6.72	27.00	24.91	0.37	0.41
6.33	5.81	6.64	6.61	21.50	24.00	0.41	0.41
6.24	5.77	6.95	6.92	19.40	21.25	0.38	0.36
6.12	4.91	6.77	6.44	17.00	26.20	0.36	0.39
6.30	5.13	8.26	6.23	26.75	21.50	0.40	0.38
5.57	5.80	8.08	7.16	24.40	26.00	0.37	0.43
6.92	5.94	6.88	5.92	22.80	23.00	0.35	0.40
6.14	6.09	6.53	6.87	19.40	28.00	0.40	0.37
6.32	5.59	6.03	6.44	17.80	23.25	0.42	0.32
6.38		7.41		24.60		0.37	
6.32		6.39		29.20		0.40	
6.16		5.93		22.80		0.42	
5.80		6.50		24.20		0.44	

Table 3.2: Vascular data values for the young 6-month cohort

The above data table shows raw data values analysed for Collagen IV measurements; vessel width, % area stained and number of vessels and Smooth muscle actin analysis; % area stained.

Vascular data for the Aged 4-month cohort							
Collagen IV						Smooth muscle actin	
Vessel width (µm)		% area stained		Number of vessels		% area stained	
Normotensive	Hypertensive	Normotensive	Hypertensive	Normotensive	Hypertensive	Normotensive	Hypertensive
6.00	6.88	2.60	5.74	17.88	21.70	0.40	0.48
7.30	6.45	3.40	3.35	22.25	25.38	0.34	0.35
6.26	5.51	3.49	3.45	20.25	22.00	0.13	0.34
6.53	7.29	1.98	5.67	19.88	21.63	0.26	0.61
6.59	7.06	2.07	5.22	20.25	23.25	0.44	0.56
7.51	7.09	1.84	3.81	18.63	17.50	0.33	0.34
6.47	6.80	4.77	5.04	22.25	22.88	0.20	0.35
6.57	6.66	4.15	5.82	20.00	24.88	0.31	0.36
7.36	6.41	4.81	3.65	19.50	22.88	0.43	0.51
	6.58		3.38		25.63		0.36
	7.00		4.19		28.00		0.58

Table 3.3: Vascular data values for the Aged 4-month cohort

The above data table shows raw data values analysed for Collagen IV measurements; vessel width, % area stained and number of vessels and Smooth muscle actin analysis; % area stained.

3.3.4. Prolonged hypertension caused structural alterations to the endothelial blood-brain barrier

To further investigate the endothelium, the structure of the endothelial BBB was assessed and in the majority of cohorts found to be unaltered, apart from hypertensive animals within the young 6-month cohort. Claudin-5 a tight junction marker was used to assess the intensity and density of protein expression. Staining was found to be similar to that of collagen IV, labelling both large and small vessels, clearly outlining the vasculature structure.

In the young 4-month cohort there was no significant difference in the intensity ($|t|= 0.25$, $df=27$, $p=0.80$, Figure 3.4(a) H, Table 3.4) or density ($p= 0.63$, Figure 3.4(b) A, Table 3.4) of claudin-5 in hypertensive animals when compared to normotensive. However, claudin-5 expression in hypertensive animals from the young 6-month cohort was found to be significantly increased in intensity of staining when compared to normotensive animals ($|t|= 3.77$, $df=21$, $p<0.01$, Figure 3.4(a) G, Table 3.5). Whilst vessels from the hypertensive animals were intensely stained, there was no significant difference in the density of claudin-5 staining when compared to normotensive animals ($p=0.10$, Figure 3.4(b) B, Table 3.5). In a previous experiment, vessels stained with collagen IV were found to have a string-like morphology; therefore vessel width was measured using claudin-5. In agreement with previous collagen IV findings, the vessel width was also was found to be decreased in hypertensive animals as compared to normotensive, when measurements were made using claudin-5 as a marker ($D= 0.34$, $p<0.01$, Appendix B.4). To further investigate these alterations, the effect of hypertension on the expression of tight junction association protein ZO-1 was examined. ZO-1 tethers

claudin-5 to the cytoskeleton and altered expression could represent structural loss of the BBB. However, there was no significant difference in the density of ZO-1 staining, in hypertensive animals when compared to normotensive (p=0.22, Appendix A.3), suggesting that claudin-5 may be more tightly clustered or redistributed but still structurally linked to the cytoskeleton.

In turn, the distribution of claudin-5 was also investigated in the aged 4-month cohort. Similar to findings in the young 4-month cohort, there was no significant difference in the intensity ($|t|= 1.89$, $df=18$, $p= 0.08$. Figure 3.4(a) I, Table 3.6) or density (p=0.32, Figure 3.4(b) C, Table 3.6) of claudin-5, in hypertensive animals when compared to normotensive. Therefore, structural alterations in claudin-5 expression were only found in hypertensive animals from the young 6-month cohort, in which vascular structural alterations had occurred.

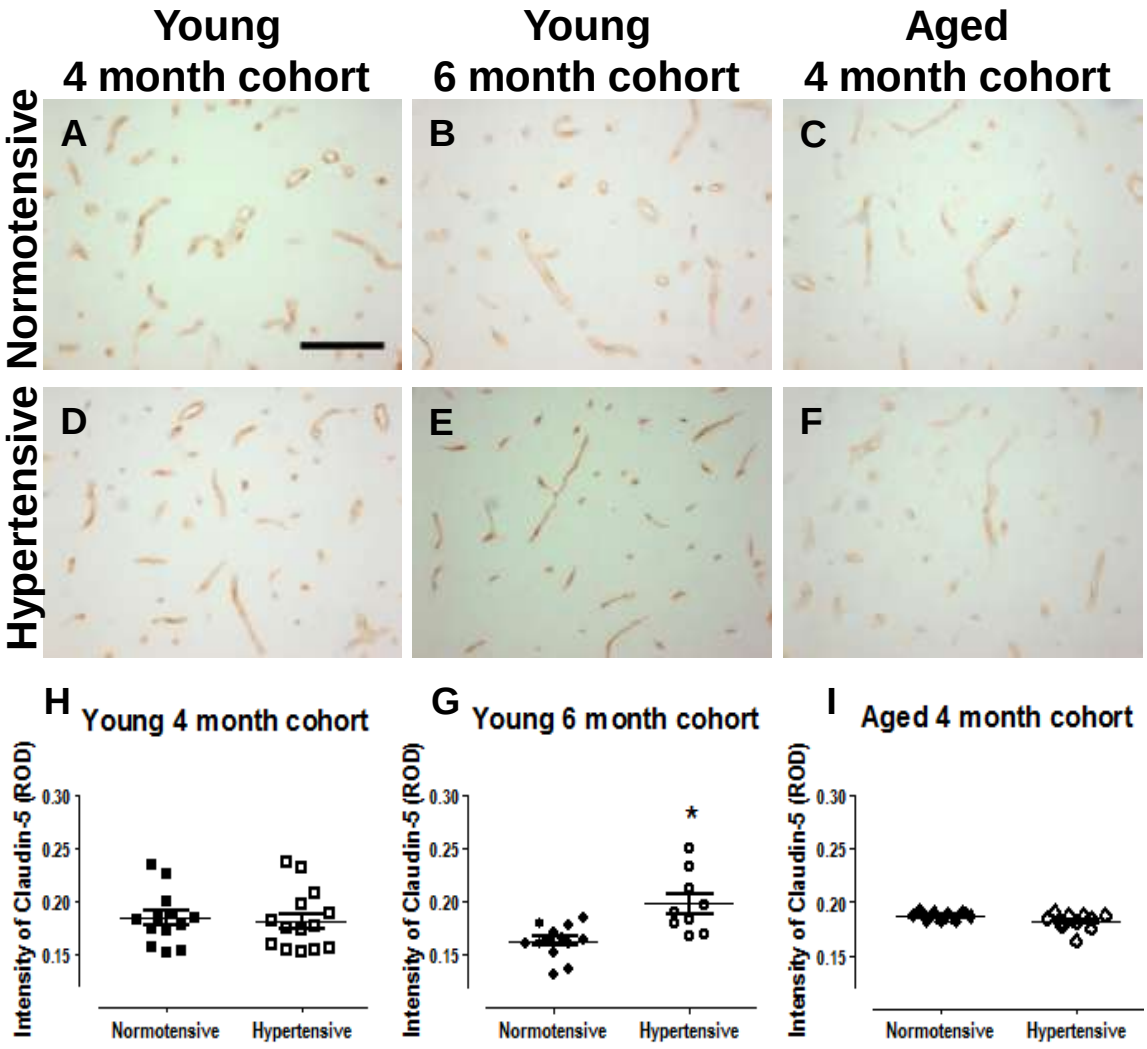

Figure 3.4a: Assessment of the endothelial blood-brain barrier

Representative images of tight junction protein claudin-5 in normotensive (A, B, C) and hypertensive (D, E, F) animals from each cohort. In the young 4-month cohort, there was no difference in the intensity of claudin-5 staining between normotensive (A, H) and hypertensive (D, H) animals. However, in the young 6-month cohort, claudin-5 staining was significantly increased in hypertensive (E, G) when compared to normotensive (B, G) animals. On the other hand, in the aged 4-month cohort there was no difference in the intensity of claudin-5 in normotensive (C, I) and hypertensive (F, I) animals. Graphs show mean ± SEM. Significance *=$p<0.01$ hypertensive vs normotensive.

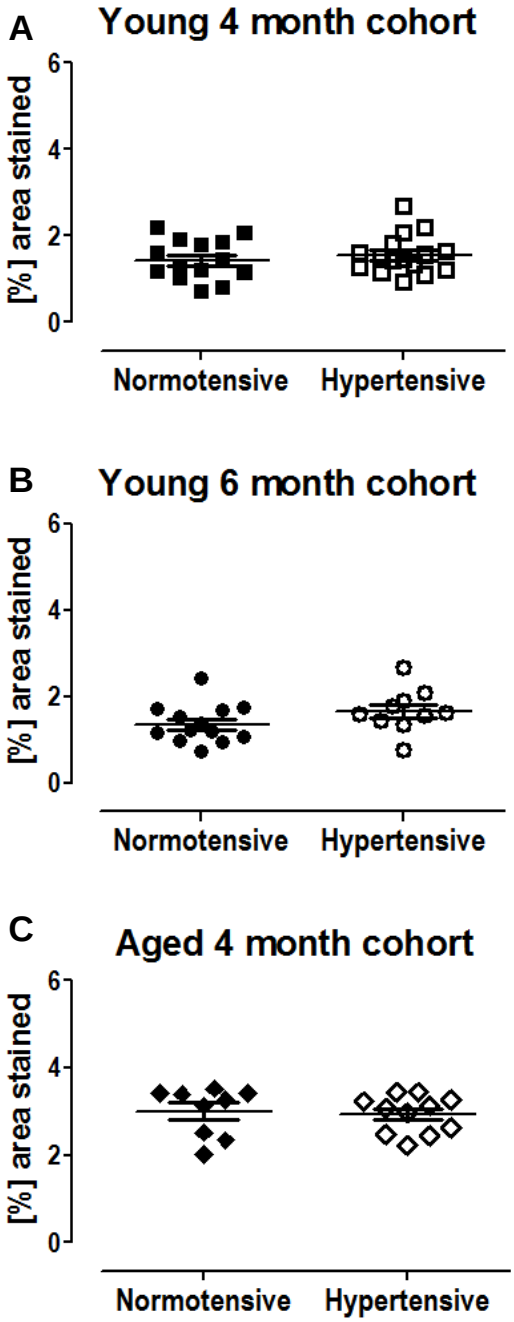

Figure 3.4b: Assessment of the endothelial blood-brain barrier
There was no significant difference in the density of Claudin-5 staining in hypertensive animals when compared to normotensive from the young 4 month (A), 6 month (B) or aged 4 month (C) cohort.

Claudin-5 data from the Young 4-month cohort			
Intensity (ROD)		% area stained	
Normotensive	Hypertensive	Normotensive	Hypertensive
0.20	0.15	2.20	2.07
0.15	0.19	1.03	0.94
0.17	0.19	2.06	2.20
0.18	0.23	1.26	1.56
0.15	0.15	3.85	1.52
0.18	0.16	1.90	1.27
0.23	0.20	0.72	1.32
0.17	0.16	1.22	1.41
0.16	0.16	1.61	2.67
0.22	0.22	1.16	1.45
0.18	0.17	0.81	1.19
0.19	0.18	1.17	1.81
	0.24		1.63
	0.21		1.59
	0.17		1.15
	0.15		1.10

Table 3.4: Claudin-5 data values for the young 4-month cohort

The above data table shows raw data values analysed for the intensity and density measurements for the young 4-month cohort from Claudin-5 immunohistochemistry.

Claudin-5 data from the Young 6-month cohort			
Intensity (ROD)		% area stained	
Normotensive	Hypertensive	Normotensive	Hypertensive
0.16	0.17	0.97	1.43
0.14	0.20	0.73	2.67
0.13	0.19	1.21	1.61
0.16	0.25	1.05	1.89
0.15	0.21	1.52	1.77
0.17	0.23	1.36	1.54
0.16	0.18	1.18	1.57
0.18	0.18	1.67	0.75
0.17	0.19	1.70	1.32
0.18		2.40	
0.16		0.94	
0.18		1.73	
0.16		1.15	

Table 3.5: Claudin-5 data values for the young 6-month cohort

The above data table show raw data values analysed for the intensity and density measurements from the young 6-month cohort for Claudin-5 immunohistochemistry.

Claudin-5 data from the Aged 4-month cohort			
Intensity (ROD)		% area stained	
Normotensive	Hypertensive	Normotensive	Hypertensive
0.19	0.19	3.41	3.44
0.19	0.18	2.02	2.23
0.19	0.19	3.13	3.23
0.18	0.18	3.53	3.44
0.19	0.19	3.42	3.28
0.18	0.18	2.52	2.48
0.18	0.18	2.35	3.14
0.19	0.18	3.27	2.13
0.19	0.18	3.42	1.98
	0.19		3.08
	0.16		1.45

Table 3.6: Claudin-5 data values for the Aged 4-month cohort

The above data table show raw data values analysed for the intensity and density measurements from the aged 4-month cohort for Claudin-5 immunohistochemistry.

3.3.5. Hypertension induced alterations to endothelial signalling

ENOS, an enzyme crucial for the production of vasodilatory NO was used as a marker of the endothelium and to provide mechanistic insight as to endothelial signalling in response to hypertension. The immunolabelling of this marker was strongly expressed and mainly observed in vessels cut in a cross-sectional orientation.

In the young 4-month cohort, there was an increased number of vessels immunopositive for eNOS in hypertensive animals when compared to normotensive ($|t|= 2.63$, df=28, $p= 0.01$, Figure 3.5, Table 3.7). In contrast, in the young 6-month cohort, there was no significant difference in the number of vessels expressing eNOS in hypertensive animals versus normotensive ($|t|= 0.82$, df=21, $p=0.42$, Figure 3.5, Table 3.7). Similar to the young 4-month cohort, hypertensive animals from the aged 4-month cohort had a significant increase in the number eNOS expressing vessels, when compared to normotensive animals ($|t|= 2.90$, df=18, $p<0.01$, Figure 3.5, Table 3.8).

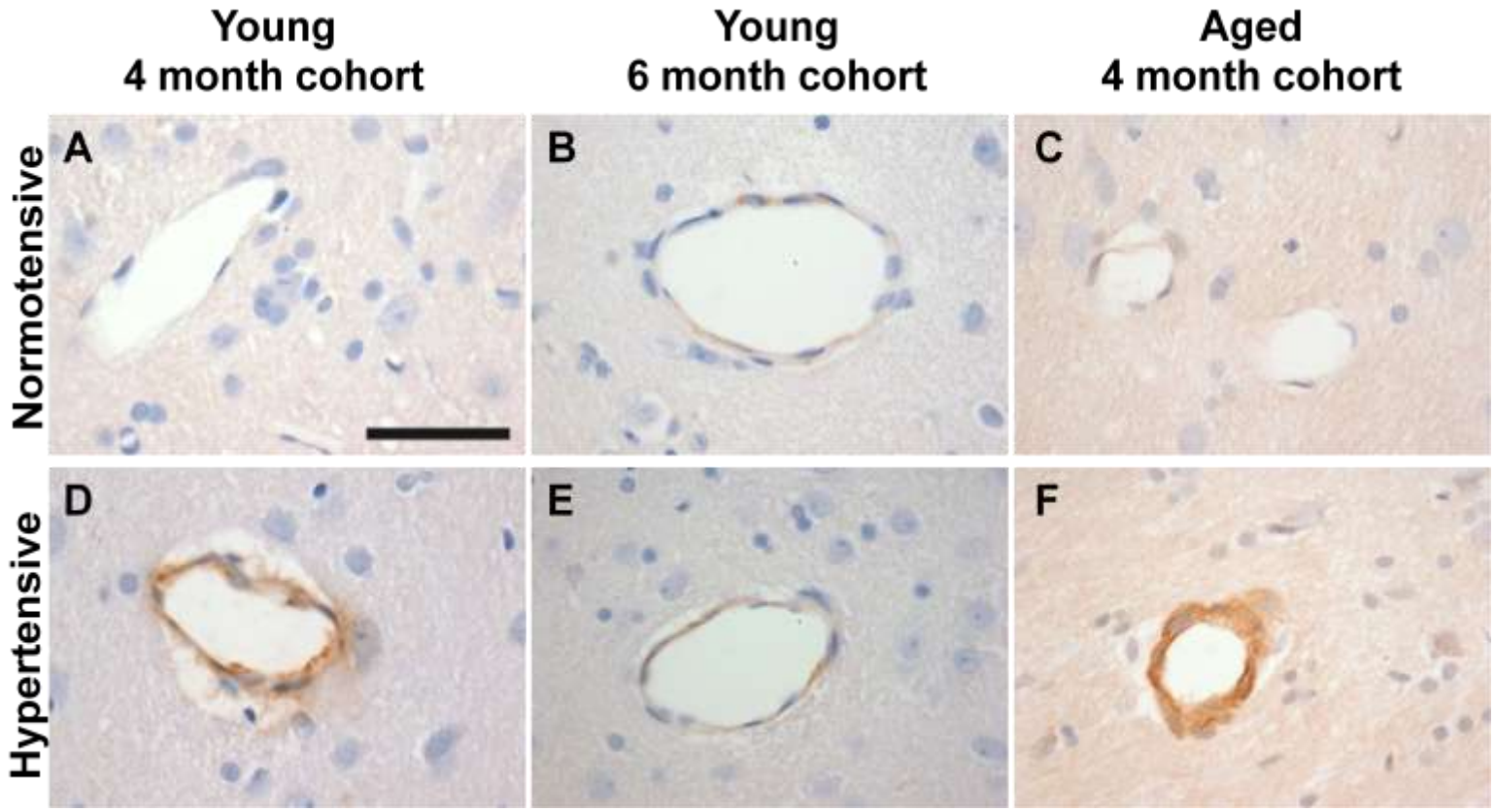

Figure 3.5a: Assessment of endothelial signalling using eNOS

Representative images of eNOS expression - eNOS (brown), counterstained with haemotoxylin (blue). In the young 4-month cohort (A, D) there was a significant increase in the number of eNOS expressing vessels in hypertensive animals (D) versus normotensive (A). No change in eNOS expression in hypertensive animals (E) from the young 6-month cohort when compared to normotensive (B). In the aged 4-month cohort (C, F) there was evidence of increased number of eNOS expressing vessels in hypertensive animals (F), compared to normotensive (C). Scale bar = 50μm.

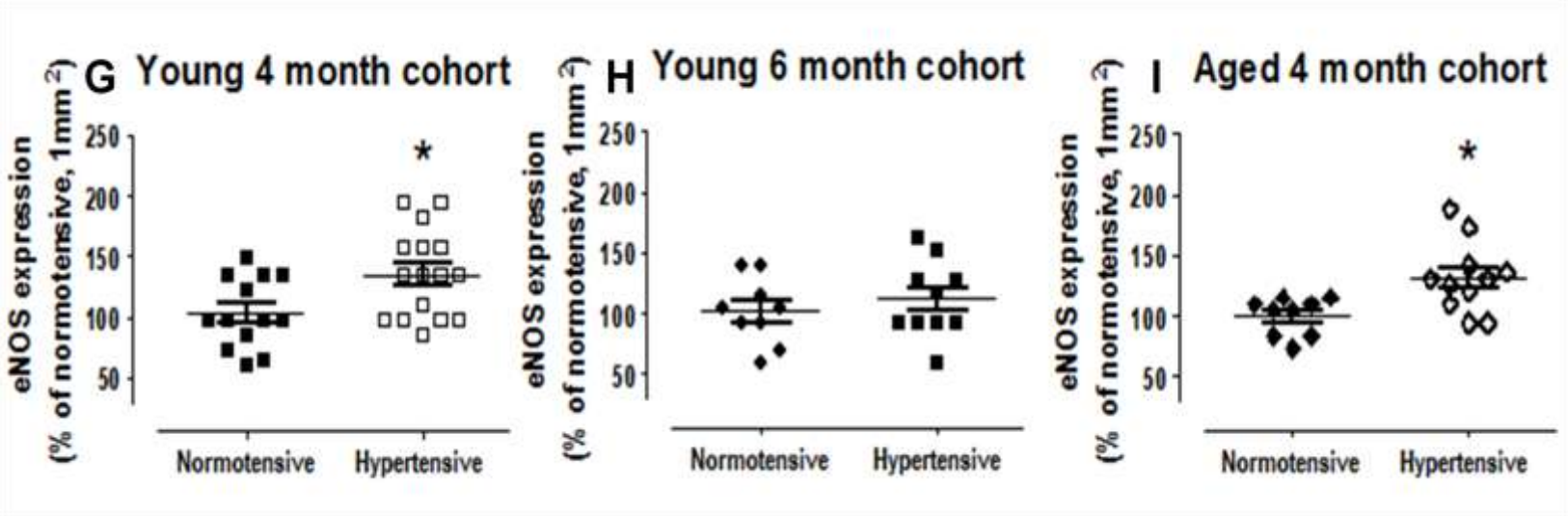

Figure 3.5b: Assessment of endothelial signalling using eNOS

Quantification of eNOS expression by counting the total number of vessels positive for eNOS within a $1mm^2$ area. In the young 4-month cohort there was a significant increase in the number of vessels immunopositive for eNOS in hypertensive animals (G) versus normotensive (G). There was no change in eNOS expression in hypertensive animals (H) from the young 6-month cohort when compared to normotensive (H). In the aged 4-month cohort there was evidence of increased eNOS expression in hypertensive animals (F, I), compared to normotensive (I). Graphs show mean ± SEM. Significance *=$p<0.02$ hypertensive vs normotensive.

eNOS data					
Young 4-month cohort		Young 6-month cohort		Aged 4-month cohort	
Normotensive	Hypertensive	Normotensive	Hypertensive	Normotensive	Hypertensive
11.0	11.0	5.0	8	8	13.5
11.0	8.0	8.0	14	10.5	12.5
12.2	7.0	6.0	10	10.5	9
8.0	11.0	8.0	8	8	12.5
6.0	11.0	9.8	11	10	16.5
10.0	16.0	12.0	5	7	9
8.0	13.0	9.0	8	11	10.5
8.0	9.0	9.0	8	11	12
8.0	13.0	12.0	11	10	13
11.0	16.0	11.0			11.5
5.0	8.0	3.0			18
6.0	13.0	13.0			
	15.0	6.0			
	9.0				
	12.1				
	7.0				

Table 3.7: Number of vessels positive for eNOS within the subcortex for each cohort

The table above shows raw count data for the total number of vessels positively stained for antibody specific for eNOS within the subcortex, although the three cohorts are presented together each was analysed separately.

3.3.6. Microglia were commonly associated with the vasculature in hypertensive animals

Iba1 a marker of microglia activation was used to investigate whether there were alterations in the inflammatory response with hypertension. Overall, there was evidence that hypertension caused vascular inflammation in the vessels of the young 6-month and the aged 4-month cohort. Microglia were observed to have a distinct cell body with surrounding processes and those found to be in association with the vasculature were counted. In the young 4-month cohort, there was no evidence that microglial cells were associated with the vasculature in any of the normotensive and hypertensive animals examined (Figure 3.6 A,D). In the young 6-month cohort, microglial cells were found to be closely associated with the vasculature in 9 out of 9 hypertensive animals, which was significantly different as compared to 2 out of 13 normotensive animals ($p<0.01$, Figure 3.6 B, E). Similarly, in the aged 4-month cohort, microglia were significantly associated with the vasculature of 11 out of 11 hypertensive animals, compared to 3 out of 9 normotensive animals ($p<0.01$, Figure 3.6 C, F). Additionally, as fully discussed in the chapter 5 the numbers of Iba1 positive microglia were counted and found to be significantly increased in the subcortex of hypertensive animals from the young 6-month ($|t|= 2.88$, $df=21$, $p<0.01$: Figure 5.9H) and aged 4-month cohort ($|t|= 2.70$, $df=18$, $p=0.01$: Figure 5.10H) but not in the young 4-month cohort ($|t|= 1.16$, $df= 27$, $p=0.26$ Figure 5.8H).

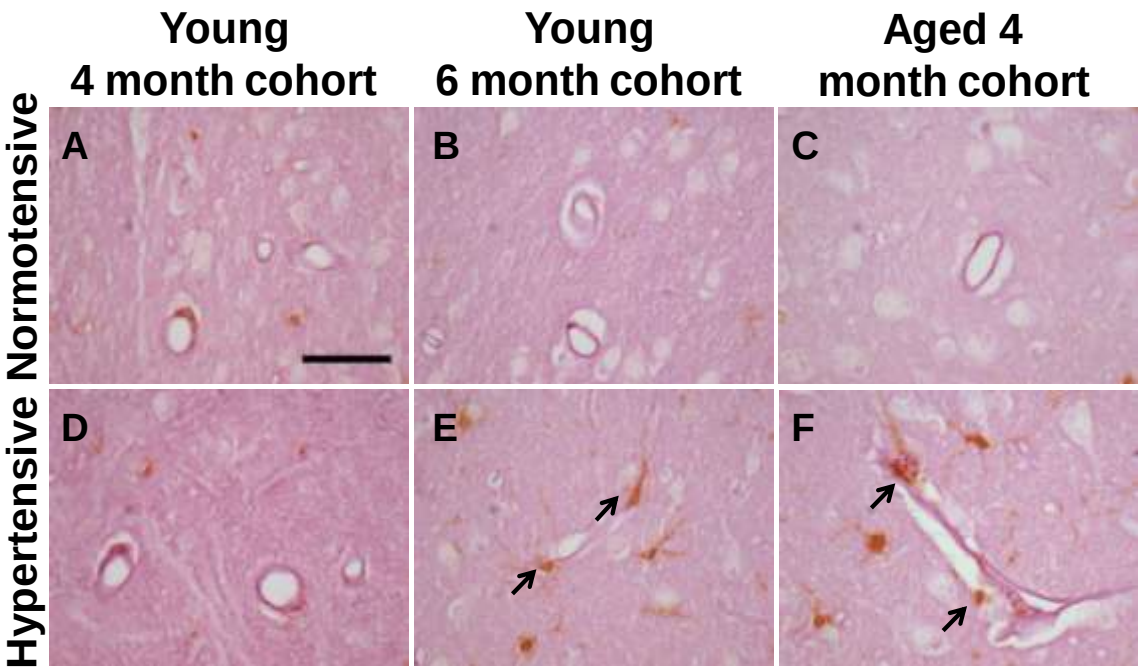

Figure 3.6: Assessment of microglia

Representative images of the microglial association with the vasculature, stained with microglial antibody IBA1 (brown) and vasculature counterstained with periodic acid schiffs reagent, in normotensive (A, B, C) and hypertensive (D, E, F) animals from each cohort. There was no evidence of microglia associated with the vasculature of normotensive (A) and hypertensive (D) animals of the young 4-month cohort. In the young 6-month cohort microglia were located in close association and contacting the vasculature of hypertensive animals (E), which was significantly different when compared to normotensive (B). The same was observed in the aged 4-month cohort, with a significant number of microglia associated with the vasculature of hypertensive animals (F) when compared to normotensive (C). Scale bar= 50µm

3.3.7. Vascular protein levels

3.3.7.1. Hypertension did not alter vascular protein levels

Within this study, hypertensive animals from the young 6-month cohort provided evidence of structural alterations to multiple components of the vasculature. To investigate whether these alterations were accompanied by changes to protein levels throughout the brain, western blot analysis of a vessel enriched homogenate was carried out.

3.3.7.2. Smooth muscle actin

There was no evidence of structural alterations in smooth muscle actin in any of the cohorts examined previously. Similar results were found with protein expression, with no significant difference in the protein levels of smooth muscle actin in hypertensive animals compared to normotensive ($|t|= 0.07$, $df=13$, $p= 0.94$., Figure 3.7).

3.3.7.3. Endothelial blood-brain barrier

PECAM is located on endothelial cells and can be upregulated with endothelial proliferation or during periods of inflammation (Williams et al., 1996). There was no significant difference in protein levels of PECAM in hypertensive animals when compared to normotensive ($|t|= 0.45$, $df=13$, $p= 0.66$., Figure 3.7).

Since the structural alterations were found in endothelial tight junction protein expression in hypertensive animals, the protein levels of claudin-5 and ZO-1 were assessed. No significant difference in protein levels of claudin-5 ($|t|= 0.32$, $df=13$, $p= 0.76$., Figure 3.7) or ZO-1 ($|t|= 0.52$, $df=13$, $p=0.61$., Figure 3.7) were found in hypertensive animals versus normotensive. Thus, this data is indicative of

hypertension causing a structural cellular re-organisation in absence of alterations to protein levels.

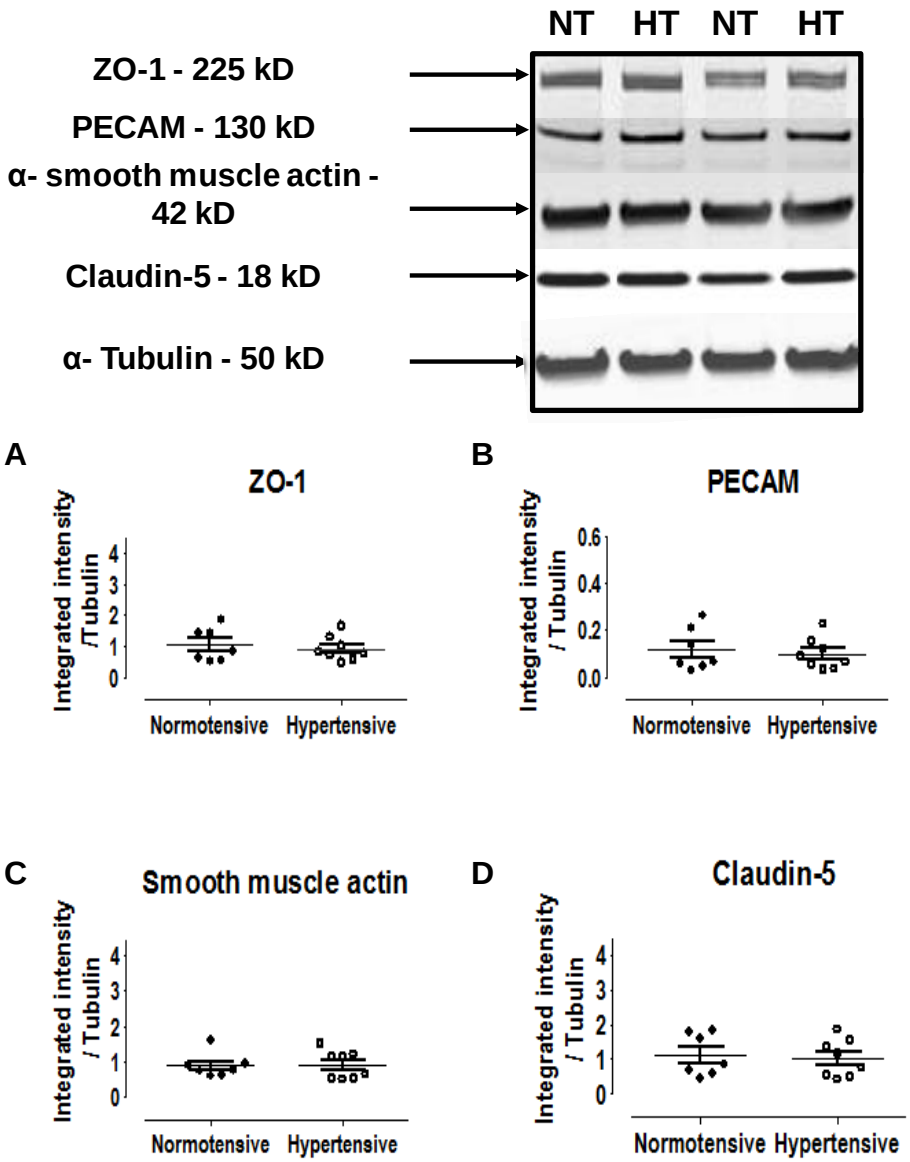

Figure 3.7: Assessment of vascular protein levels

Protein levels of vascular components were unaltered in hypertensive (HT) animals from the young 6-month cohort when compared to normotensive (NT). Western blotting for ZO-1 (A), Pecam (B), α-smooth muscle actin (C) and Claudin-5 (D) showed no significant difference in protein level between normotensive and hypertensive animals. Graphs show mean ± SEM

3.4. Discussion

Previous evidence has indicated that hypertension is associated with structural alterations to the cerebrovasculature in humans and animal models. However, due to discrepancies in the ability to model hypertension in isolation, without confounding factors, the exact mechanisms are unknown. The present study sought to investigate hypertension induced alterations to the cerebrovasculature in the brains of three separate cohorts; the young 4-month cohort, the young 6-month cohort and the aged 4-month cohort, within the subcortex, a region commonly affected by hypertension in humans. The key findings of this study were that hypertension caused subcortical vascular alterations associated with endothelial changes and an inflammatory response, which were observed in the young and aged brain. Overall, the findings within this study are suggestive that hypertension, when modelled in a controlled sustained fashion with the use of genetically matched controls, does lead to alterations in the structure of the cerebrovasculature, particularly endothelial related, albeit relatively subtle.

3.4.1. Blood pressure in the Cyp1a1 Ren2 rat model

Dietary induced hypertension by the addition of I3C was found to cause a sustained and gradual increase in systolic blood pressure levels of animals fed an I3C inclusive diet, in each of the cohorts studied. The cohorts were analysed separately, as although similar methodology of dietary induction was used, they were carried out at different times, in different animals units and ran as three distinct cohorts. However, it does need to be discussed that within the aged cohort around week 8 there is a sudden increase in blood pressure of both animals fed a control diet and those fed a hypertensive inducing diet. This was unfortunately attributed to a

technical fault within the animal unit, in which the air conditioning unit broke one evening and the animals were immediately rehoused. The animals were extensively examined by the vet and they were all observed to have consumed their diet as normal, but this event appeared to cause a period of distress, which is reflected within their blood pressure recordings for that period. In general, the level of blood pressure generated was comparable to previous studies in this model (Huang et al., 2012; Mitchell et al., 2006). However, the profile of blood pressure is different from that of the commonly used models; the SHR (Okamoto and Aoki, 1963) and SHRSP (Okamoto, 1974; Yamori and Horie, 1977), which have been found to evoke increased blood pressure in a sudden, spontaneous manner, with the latter model causing elevations in blood pressure equivalent to that in malignant hypertension. The advantage of raising blood pressure in a gradual manner, (as in the Cyp1a1 Ren2 rat), is that it is representative of how hypertension develops in humans. In turn, the controllable nature of the severity of blood pressure in this model ensures that hypertension, rather than malignant hypertension is modelled. Commonly hypertension is referred to as the 'silent' killer, as many people are un-aware they are hypertensive, therefore modelling hypertension of a lower severity would allow the deduction of several mechanisms more indicative of the general population, which may have been missed in other models and may contribute to its role as a risk factor for the gradual developing neurodegenerative conditions (Stevens, 2008). Another advantage is that the increased blood pressure in this model can be directly compared to genetically matched, litter-mate controls, making pathological observations resulting from chronic increased blood pressure more robust. Thus down-stream

consequences of hypertension on the vascular alterations could then be studied and compared to normotensive controls.

3.4.2. Hypertension induced alterations to the cerebrovasculature

The present study investigated the structural integrity of the cerebrovasculature, examining basement membrane specific protein, collagen IV. For each antibody examined within this thesis a blank slide (which received the same immunohistochemical treatment apart from primary antibody) was contained within each run of immunohistochemical staining to ensure that specific staining was not attributed to background. There was no evidence of overt structural alterations to the cerebrovasculature in the young 4-month cohort, indicating that this duration of hypertension is not sufficient to induce vascular remodelling. However, with increased duration of hypertension in the young 6-month cohort, the vasculature was found to be markedly reduced in width in hypertensive animals only, with the vessels observed to be string-like in morphology. Even though the vessels were thinner, this was not accompanied by any alterations in the density of collagen IV. In addition, these morphological differences were not found in any of the normotensive controls within the young 6- month cohort. In the present study, the finding that hypertension did not alter vascular density of collagen IV is in agreement with immunohistochemical studies in the SHRs and SHRSPs (Bailey et al., 2011; Lin et al., 2001). On the other hand, there is evidence of increased density of collagen IV in 6 month old SHRSPs (Liebetrau et al., 2005), which cannot be exclusively attributed to hypertension as this is an age when SHRSPs commonly develop stroke like symptoms (Tagami et al., 1987; Yamori and Horie, 1977). However, the present study also found evidence of string-like vasculature in hypertensive animals of the

young 6-month cohort and this is not in agreement with findings in either the SHR (Knox et al., 1980; Nordborg and Johansson, 1980; Sabbatini et al., 2001) or SHRSP (Fredriksson et al., 1985; Tagami et al., 1987) models, which have shown evidence of basement membrane thickening, luminal narrowing and collagen deposition. Nonetheless, vascular remodelling has been found to occur in SHRs and SHRSPs as young as 15 days old, prior to the development of hypertension (Nordborg and Johansson, 1980; Okamoto, 1974). In addition, vascular remodelling in the SHRSPs has also been found shortly after the development of hypertension, with evidence indicating the SHRSPs may have constitutively smaller vessels than their WKY normotensive controls (Mies et al., 1999; Zanchi et al., 1997). In turn, a recent study carried out to examine vascular integrity in 10 month old stroke free SHRSPs found no evidence of small vessel vasculopathies, thus it is unclear whether vascular alterations documented in the SHRSPs are solely due to hypertension or due to stroke (Brittain et al., 2012).

In the present study, the lack of alterations to vascular structure with the shorter duration of hypertension indicates that this model does not have a predisposition for vascular remodelling, and that alterations with increased duration are indicative of the type of hypertension induced. The morphological alterations found in the young 6-month hypertensive animals may represent aberrant vasoconstriction with hypertension, a concept induced by Folkow et al., who proposed that chronic hypertension would lead to increased vascular resistance caused by an aberrant vasoconstrictive response leading to a form of vascular remodelling known as eutrophic (Folkow et al., 1958): when the outer and lumen diameters are decreased by cellular re-organisation, rather than deposition (Mulvany,

1999). This form of remodelling has been found in the Renin^{+}/angiotensinogen^{+} and Angiotension II infusion (ANGII) mouse models of hypertension, which induced hypertension, similar to our model, by targeting the RAAS (Baumbach et al., 2003; Moreau et al., 1997) and this form of remodelling has been observed peripherally in our model (Kantachuvesiri et al., 2001). In the present study initial mechanisms underlying decreased vessel size could be due to alterations in myogenic tone (Davis and Hill, 1999) resulting from increased pressure and may represent endothelial dysfunction. In turn, modelling acute hypertension has also been shown to induce an aberrant vasoconstrictive response, due to altered sympathetic nervous system activation (Tamaki and Heistad, 1986). Although in the present study, the functional consequences of these morphological alterations to the vasculature are not yet known, it could be proposed that they may lead to alterations in vascular resistance, blood flow and energy supply. However, it needs to be said, one limitation of the present study is that cerebral blood flow in response to hypertension was not measured. Even though one would postulate that the morphological alterations of string like vessels would decrease blood flow within the subcortex, this has not been measured and remains unknown. In turn, it is unknown how these morphological alterations would have impacted on the integrity of the vessels in the form of BBB leakiness. The only conclusion which can be gleaned from the present study is that hypertension in the young brain leads to structural alterations to the cerebrovasculature and how this impacts on the gross structural integrity of the brain is examined later within this thesis.

In addition the present study also examined the effect of hypertension on the structure of the cerebrovasculature in the aged rat brain, with the age of animals

comparable to middle age in humans. The present study found that hypertension in the aged brain leads to increased vascular density and number of vessels, in absence of the string-like vessels found previously. In general, there is limited data in aging animal models of hypertension but our findings are in disagreement with a study in 9-16 month old SHRs finding evidence of reductions in arteriolar end point density and surface anastomic connections, indicative of decreased vascular density compared to WKY (Hutchins et al., 1996). The reason for the disagreement could be due to the duration of hypertension being far longer in the SHR model than the duration induced in ours, as in the present study the animals were allowed to age naturally and induced to become hypertensive in adulthood. However, the present data is in agreement with findings in the young SHR brain with studies reporting increased numbers of vessels (Gesztelyi et al., 1993; Lin et al., 1990; Ritz et al., 2009), but these alterations have also been found in prehypertensive animals, making data concerning number of vessel in relation to hypertension difficult to interpret in the SHR model. In addition, it does need to be highlighted that there appears to be an overall decreased vascular density in both the normotensive and hypertensive aged animals compared to the young. Unfortunately, as the three cohorts were run distinct to each other with many conflicting factors the cohorts cannot be statistically compared. It is unknown if there is a difference in antibody penetration as the tissue from the aged cohort was processed in a different facility. These studies were carried out as initial characterisation studies to investigate the overall effect of hypertension on the structural integrity of the brain, future studies would need to be set up with the 3 cohorts run at the same time to remove temporal, location and methodological bias.

In the present study the increased number of vessels in the aged brain may be a response to hypoxia, as previous studies modelling ischemia/hypoxia have shown an angiogenic response with an increased number of vessels, specifically within the thalamus (Hayward et al., 2011). Additionally, individuals with various forms of dementia have been found to have an increased expression of angiogenic growth factors (Tarkowski et al., 2002). However, the present study did not address regional atrophy of the brain in response to hypertension, which may lead to an over-representation of the number of vessels, but work carried out by Dr P Holland in these animals found no evidence of regional atrophy using T2 MRI (P Holland et al., in preparation, Appendix I). Studies carried out in aged Fischer F344 animals have reported brain atrophy at 24 months of age (Hajdu et al., 1990) and studies in the SHR model have reported that hypertension can induce atrophy in the young brain (Tajima et al., 1993). Nonetheless, these previous findings have assessed atrophy in post-fixed tissue. The fixing of tissue brain tissue can lead to shrinkage, therefore, a true indication of atrophy would need to be assessed using in-vivo imaging (Stowell, 1941). Moreover, the findings in the present study indicate that age in combination with hypertension exacerbates the effects of hypertension, as there was no evidence of vascular alterations in the young cohort, modelled for a similar duration of hypertension.

One limitation of the present study, is that only one component of the basement membrane was measured. The basement membrane, is a key component of the vasculature, providing both structural support and a substrate for cellular interactions (Perlmutter and Chui, 1990; Schwarzbauer, 1999). Collagen IV is highly abundant within the lamina densa of the basement membrane (Perlmutter and Chui,

1990), forming the structural backbone onto which other components bind (Schwarzbauer, 1999). This endows the structure of collagen IV crucial for the function and maintenance of the basement membrane (Poschl et al., 2004), an intermediate layer juxtaposed to astrocytic end-feet and endothelial cells, whose structural alterations can impair connections between communicating cells (Farkas et al., 2001).Therefore, in the present study it is unknown if there is deposition of other components i.e., laminin or fibronectin but, as collagen IV is the most abundant and forms the backbone of the basement membrane, any overt morphological alterations would likely be mimicked by other components to which it anchors, or one would expect to have evidence of separation of the basal lamina, which was not observed with collagen IV staining in any of the animals, in any of the cohorts. Additionally the 2A subunit of Collagen IV was examined as this has been found to be commonly affected in adults with small vessel disease (Dichgans and Hegele, 2007).

Overall, these studies have provided evidence of hypertension induced subtle structural alterations to the vasculature, which may lead to alterations in CBF within the subcortical thalamic region of the brain. These vascular alterations may impair vascular function within this region, leading to alterations between cortical-subcortical regions of the brain and may lead to alterations to the overall structural integrity of the brain, including the WM, which is sensitive to alterations in blood flow. The present study represents alterations to the general structural integrity of the vasculature in the young and aged brain without discrimination between different types of vasculature.

3.4.3. Hypertension does not induce structural alterations to the large arteries and arterioles

To follow on from the assessment of the basement membranes, alterations in the large arteries and arterioles were examined. Smooth muscle actin was used to define these vessels as it is absent in small arterioles and capillaries (Drake and Iadecola, 2007). Overall, the present study found that hypertension did not alter the density of smooth muscle actin, suggestive of no overt vascular remodelling in larger arteries and arterioles. These findings were also confirmed with no evidence of alterations in smooth muscle actin protein levels (Figure 3.7). The findings in the present study are in disagreement with both the SHR (Knox et al., 1980) and SHRSP (Bailey et al., 2011; Lin et al., 2001) models providing evidence of smooth hypertrophy at around 3 months and 12-16 weeks respectively. As described previously, it is unclear as to whether these results in the SHRs and SHRSPs are exclusively due to hypertension, as the large arteries in particular, of both SHRs (Nordborg and Johansson, 1980) and SHRSPs (Johansson, 1984; Zanchi et al., 1997) show remodelling as young as 15 days. Alternatively, the level and method of inducing hypertension in the present study may not be a sufficient insult to alter the smooth muscle layers and it remains to be said that only a crude investigation of the large arteries and arterioles was carried out. However, the findings are suggestive that hypertension in our model, targets the smaller vasculature of the brain. This is in agreement with previous findings in the SHR model, which shows vascular remodelling occurs more often in small arterioles and capillaries than the large arteries and arterioles proposed to be more susceptible due to a lack of anatosomes (Sabbatini et al., 2001).

3.4.4.1. Hypertension induced alterations to the endothelial blood-brain barrier

To determine whether alterations in the vasculature may be accompanied by BBB changes the integrity of the endothelial BBB was investigated by examining the integrity of tight junction protein claudin-5. It was found that hypertension did not induce alterations in claudin-5 expression, in the young 4-month cohort, in the absence of alterations to cerebrovascular structure. Interestingly, in the young 6-month cohort at a duration when the vascular structure was altered, hypertension caused an increased expression of claudin-5 in absence of alterations to the expression of claudin-5 tethering protein ZO-1 (Figure 3.7). In addition, there was no evidence of alterations to protein levels of either claudin-5 or ZO-1 (Appendix B.1). This may represent a redistribution of claudin-5 rather than a change in protein levels, due to the alterations in vascular structure, leading to a clustering of tight junctional strands, similar to the SHRSPs showing a redistribution of endothelial tight junctional proteins associated with a loss in endothelial polarity (Lippoldt et al., 2000). There is also evidence in the SHRSPs that there is a decrease in claudin-5 levels prior to hypertension, which may predispose them to endothelial BBB alterations (Bailey et al., 2011). However, findings in the SHRs are dissimilar from ours showing no evidence of alterations in tight junction proteins: ZO-1, occludin and claudin-5 with hypertension alone and the combined insult of hypertension with MCAO (Hom et al., 2007b).

It was hypothesized that in the ageing brain alterations in the BBB would be exacerbated with hypertension, but the present study did not find evidence of alterations in Claudin-5 expression in aged hypertension animals when compared to

normotensive controls. The lack of alterations in Claudin-5 expression observed in the aged brain may indicate that this severity of hypertension is not sufficient to cause alterations, in agreement with the SHR literature described above (Hom et al., 2007a) and that these alterations observed in the young 6-month cohort may be a response to alterations in vascular structure.

Taken together the results in the inducible transgenic rat model suggest that the BBB is modestly altered after a prolonged period of hypertension and in association with morphological vascular alterations. The function of the endothelial BBB is critical, limiting the flow of harmful stimuli into the brain. A link between endothelial dysfunction, BBB disruption and vascular inflammation has been proposed (Saavedra et al., 2011; Wolburg and Lippoldt, 2002; Yenari and Han, 2006). In turn, alterations to intracellular ionic concentrations have also been associated with alterations to the structure of the endothelial BBB (Huber et al., 2001; Wolburg and Lippoldt, 2002). The tight junctional protein claudin-5 was chosen in the present study as evidence has shown that claudin-5 is integral to the formation of the endothelial BBB. The findings within the study may be a response of the vasculature to sheer stress, since tight junctional proteins have been observed to redistribute and cluster during periods of sheer stress, strengthening the BBB (DeMaio et al., 2001; Yoshida et al., 1995). In turn, the increased expression of inflammatory molecules such as Interleukin-1, which can be released from microglia have been found to cause a de-polymerisation of the cytoskeleton leading to a redistribution of tight junctional proteins (Eisenhut and Wallace, 2011). However, it cannot be excluded that morphological alterations to vascular structure are not a causal factor in the increased expression of claudin-5. Although it remains to be

defined, it can be postulated that these alterations in claudin-5 expression may impair transcellular flow and could even prevent the entry of nutrients into the brain or impair the removal of harmful stimuli.

A limitation of the present study is that it did not measure whether these alterations to endothelial BBB structure caused BBB leakage. Using different approaches such as horse radish peroxides, the BBB has been found to be impaired in both the SHR and SHRSP models. However, BBB leakage is not found in every vessel and is proportional to the severity of hypertension evoked, being more readily observed in malignant hypertension. Notably, the level of hypertension induced in this inducible model is a lower severity of the SHRs (Knox et al., 1980; Ueno et al., 2004a; Ueno et al., 2004b) and SHRSPs (Fredriksson et al., 1985; Fredriksson et al., 1987; Fredriksson et al., 1988), therefore this overt BBB leakage may not be expected. Another limitation of this study is that it only examined one tight junction protein. Although, claudin-5 is imperative for the formation of the endothelial BBB it is unknown what effect alterations to other components of the BBB may have in this study. Nonetheless, the pathological effect of these alterations is investigated in the following experiment, examining endothelial signalling and vascular related inflammatory response.

3.4.5. Hypertension induced alterations to endothelial signalling

Previous experiments in the present study had provided evidence of morphological alterations to vascular structure and evidence of endothelial BBB alterations. Due to links between disruption of endothelial signalling and vascular remodelling, alterations in endothelial signalling were investigated by the expression

of the endothelial enzyme, eNOS, crucial for the production of vasodilatory NO. ENOS expression was analysed by counting the number of vessels, which were immunopositive for the eNOS antibody. Interestingly, eNOS was found to be increased in vessels of hypertensive animals from the young 4-month cohort in absence of any structural alterations. However, there was no difference in eNOS expression between normotensive and hypertensive animals in the young 6-month cohort. The data suggests alterations in endothelial signalling may precede alterations in vascular structure in the young brain. In agreement with the present findings, global investigation of eNOS expression has been found to be decreased in SHRs and SHRSPs, shortly prior to vascular remodelling, with a more severe decrease during malignant hypertension in the SHRSP model (Chou et al., 1998; Hojná et al., 2007; Jesmin et al., 2007). Nonetheless, there has been evidence of increased eNOS expression in regions of the brain crucial for blood pressure control, linking eNOS to the maintenance of hypertension (Paton et al., 2007a). Overall previous studies demonstrate that the activity of eNOS is dependent on the region of the brain and also severity of hypertension induced. However, the SHR and SHRSP data is difficult to interpret, as the WKY normotensive control strain has been found to exhibit decreased expression of eNOS with age (Chou et al., 1998).

The present study also examined eNOS expression in the aged 4-month cohort and found that hypertensive animals had increased number of vessels positively expressing eNOS when compared to normotensive controls. Unlike the young brain this increased eNOS expression with hypertension is accompanied by an increase in vascular density. The exact cause for the increased number of vessels is unknown within this study, but eNOS can be activated by proangiogenic VEGF

(Bouloumie et al., 1999; Drummond et al., 2000; Förstermann, 2010). VEGF not only increases eNOS production but can cause an increased vascular permeability and release of proinflammatory mediators, prior to endothelial proliferation and angiogenic response (Croll et al., 2004). Therefore the increased expression of eNOS in the aged brain coinciding with subtle vascular alterations may represent the eNOS pathway as an important pathway early in hypertensive related alterations..

However, the present study did not investigate the number of vessels with age alone. It is possible that increased eNOS expression with hypertension in the aged brain is functioning to prevent loss of vessels. Studies have shown that eNOS and the generation of NO prevents apoptosis (Burger et al., 2006; Dimmeler et al., 1999). Therefore, the aged normotensive animals may have a loss of vasculature density, which increased eNOS expression is preventing.

All in all, the present study highlights alterations in endothelial signalling as an initial mechanism of hypertension in the brain. Endothelial function is crucial for providing energy to the brain, allowing the appropriate entry on ions and preventing the entry of harmful stimuli, by correct signal transduction between the blood and brain. Increased blood pressure, alterations in blood flow, sheer stress and oxidative stress promote the release of eNOS (Paravicini and Touyz, 2006; Sase and Michel, 1997; Zhang et al., 2006). Hypertension has been associated with dysfunction of endothelial signalling, leading to the production of ROS and inflammation (Endemann and Schiffrin, 2004). In turn, alterations in endothelial signalling have been linked to an aberrant vasoconstrictive response, which could lead to altered ion channel function, recruitment of inflammatory cells and production of ROS (Endemann and Schiffrin, 2004; Faraci et al., 2005; Vanhoutte, 2009). Therefore, it

could be postulated that in the present study, increased blood pressure activates a signalling cascade promoting the endothelium to release eNOS, in an aim to lower blood pressure, reduce the possibility of sheer stress and alterations in blood flow, in both the young and aged brain. With increased duration of hypertension, the vasculature undergoes morphological alterations and eNOS expression is dampened, which may be representative of endothelial dysfunction. None the less, it is crucial to highlight that this model induces hypertension through an increase in peripheral RAAS system, which leads to the increased expression of a well-known hypertension related culprit Angiotension II. It is not clear whether Angiotensin II crosses the BBB in this model, but studies have found Angiotensin II to stimulate the production of eNOS (Paton et al., 2008). Since eNOS is not constitutionally increased in this model, the alterations observed cannot be exclusively due to model specific induced Angiotensin II levels and are more likely due to hypertensive induced mechanisms.

Studies have shown that activation of the endothelium and astrocytes leads to an increase in intracellular Ca^{2+}- which can lead to alterations in endothelial signalling pathways, causing phosphorylation of the cytoskeleton and opening of tight junctions (Huber et al., 2001; Wolburg and Lippoldt, 2002). Alterations in ionic influx could lead to an increased inflammatory response, through Ca^{2+} signalling. In turn, increase intracellular Ca^{2+} has traditionally been associated with vasodilation, but can also lead to vasoconstriction during periods of decreased K^{+} channel activity (Filosa et al., 2004) (Mulligan and MacVicar, 2004). Even though the cause and consequence of these alterations in endothelial signalling is not fully known, the present study provides evidence of the initial alterations in signalling mechanisms

prior to cerebrovascular remodelling and a possible therapeutic window of intervention.

3.4.6. Alterations in vascular structure are associated with increased inflammatory response

Microglia are recognised to be sensitive to changes in the brain/cellular homeostasis, thus this study sought to determined whether localised changes in microglia may accompany vascular alterations by examining the association of microglia with the vasculature. In support of this, whilst no changes were observed in the young 4-month hypertensive animals, increased microglia were observed to be localised to the vasculature of hypertensive animals from the young 6-month cohort. Not only was there evidence of increased numbers of microglia associated with the vasculature, they were subsequently found to be increased in number within the region as a whole, when compared to normotensive animals. Additionally the location of microglia was also examined in the aged brain, finding that hypertensive animals from the aged 4-month cohort had increased number of microglia associated with vasculature and similar to the young 6-month cohort, there were significantly increased numbers of microglial within the region as a whole, when compared to normotensive animals. The findings in present study are in agreement with early histological studies in both the SHR and SHRSP, providing evidence of increased numbers of perivascular microglia and macrophages surrounding remodelled vasculature (Hazama et al., 1975; Knox et al., 1980). In parallel, an increased expression of other pro-inflammatory markers such as JAM-1 and an increased number of leukocytes have been found in the SHR models, prior to the development of hypertension and during developed hypertension (Waki et al., 2011), providing

evidence that vascular inflammation plays a role in the development of hypertension in the SHR model. Collectively, evidence from the SHR and SHRSP models suggests that the severity of hypertension is a key factor in vascular inflammation. Comparison of alterations between the SHR and SHRSP models have found that although there is evidence of vascular inflammation in the SHR model, this is far less severe when compared to age matched SHRSPs (Lin et al., 2001).

The present study provides evidence that vascular inflammation occurs with increased severity of hypertension in association with vascular remodelling. Microglia were examined as they are the brain's resident innate immune cell, which are extremely sensitive to alterations in their microenvironment (Ransohoff and Perry, 2009). These cells are continually active, scanning the brain for alterations to their environment and are able to promote proliferation, migration and phagocytosis in other microglial cells, in response to alterations (Davalos et al., 2005; Nimmerjahn et al., 2005). However, during injury the increased expression of microglia can be both neuroprotective, due to the removal of harmful material and also neurodegenerative, due to the release of pro-inflammatory mediators (Meda et al., 1995; Tan et al., 1999).

The recruitment of microglial cells can be stimulated by glia-vascular Ca^{2+} signalling, though astrocytes, which due to their position receive signals from neurons and blood vessels. Evidence has shown that during endothelial and astrocytic activation there is an increase in intracellular Ca^{2+} concentration, which can promote alterations to endothelial signalling, phosphorylation of the cytoskeleton and opening of the endothelial BBB (Huber et al., 2001; Wolburg and Lippoldt, 2002). In turn, periods of increased eNOS expression have been associated with the

production of ROS, which themselves can release factors, promoting vasoconstriction but can also lead to increased inflammatory signalling and thus recruitment of inflammatory cells (Paton et al., 2006; Vanhoutte, 2009). Moreover, the increased numbers of microglia may themselves lead to alterations to vascular structure by the production of TNFα, which can lead to increased expression of prostaglandin E2 (Engblom et al., 2002; Schiltz and Sawchenko, 2003) and aberrant vasoconstriction (Vanhoutte, 2009), as found in the young 6-month cohort. Conversely, the recruitment of microglia in the present study may be due to alterations of intracellular ions, paracellular flow or nutrient supply to the brain, as a result of vascular structural alterations. Thus, the present study indicates that hypertension in the inducible model provides evidence of a link between alterations in endothelial signalling, vascular remodelling and inflammation.

Limitations of the present study are that only one inflammatory marker was used to examine microglia. In turn, the study did not take into account other inflammatory cells such as leukocytes, which have been found to be highly upregulated in the SHRSPs (Takemori et al., 2000), but chose to examine microglia as a cellular component of the neurovascular unit. In general, the present study provides a link between alterations in endothelial signalling, vascular remodelling and inflammation in the young and aged brain. The exact aetiology and function of increased microglia in the brain is unclear in the present study but provides a crucial therapeutic target and also an appropriate intervention period. It has been shown in previous animal studies that anti-hypertensive treatment, which decreases the inflammatory response prevents vascular remodelling when compared to non-treated hypertensive animals (Ando et al., 2004). However, the efficacy of the hypertension

treatment in the brain is related to how strongly they cross the endothelial BBB. The present study highlights the importance of endothelial signalling and vascular inflammations and identifies possible therapeutic potential.

3.4.7. Hypertension induced structural alterations to the vasculature without alterations to protein levels

The present study found alterations to the structural integrity of the cerebrovasculature, but it is unknown if these changes are due to cellular re-organisation or alterations to protein levels. To investigate the link between structural alterations and protein levels, a vessel enriched homogenate was used to investigate the levels of several vascular proteins including, smooth muscle actin, endothelial specific PECAM, claudin-5 and ZO-1 in the young 6-month cohort, which had previously been shown to have several vascular alterations. It was found that there was no evidence of alterations in protein levels in any of the proteins examined. These findings are in agreement with previous findings in the SHR model, which found no alteration in the protein expression of actin, ZO-1 and claudin-5 using western blot method for identification (Hom et al., 2007a). However, these findings may be in part due to the detection method, with western blot being a semi-quantitative method requiring a difference of more than 10% to reveal a significant difference. In house within the lab, liquid chromatography- mass spectrometry (LC-MS) was used as a more sensitive approach; however this required a vessel enriched homogenate from the whole brain and still revealed small levels of the endothelial BBB proteins. In turn, another method which could have been used would have been enzyme-linked immunosorbent assay (ELISA), but many of the ELISA kits available

to investigate vascular proteins only allow the detection of one protein at a time, meaning the use of a large volume of sample per marker. Overall, the findings in this study indicate that hypertension leads to structural cellular reorganisation or a redistribution but does not cause overt alterations in protein expression.

3.4.8. Summary of vascular alterations

Overall, the present study provided evidence that hypertension in isolation leads to alterations in vascular structure, endothelial signalling and an inflammatory response. These changes occurred at levels of blood pressure more akin to humans prior to the development of malignant hypertension. From the present study it would appear that alterations in endothelial signalling are the initiating factor leading to structural and inflammatory alterations. As in humans, there is also evidence that the effects of hypertension are present within the aged brain. This study whilst providing a basis to show structural changes in the vasculature, in response to modest hypertension associated with localised cellular response has several limitations (discussed previously), of these the mechanism leading to these changes remain to be explored. In the next chapter alterations in gene expression in response to hypertension will be investigated as a means to probe alterations in functional pathways.

4. The effect of hypertension on gene expression and functional gene pathways

4.1. Introduction

In humans, the majority of studies examining alterations in gene expression have focused on genes that may predispose individuals to hypertension, rather than those altered in response to hypertension. Generally, studies have found that hypertensive individuals have altered gene expression within the RAAS pathway, ion transport and endothelial NO signalling pathways (Levy et al., 2009; Salvi et al., 2012). Recent improvements in microarray technology have allowed gene expression studies in animals to be more robust and accessible. Although still within their infancy, to date studies in the SHR and SHRSP models have generally found alterations in the expression of genes concerning vascular inflammation, endothelial, lipid metabolism, oxidative stress, ischemic response, and novel vascular specific genes (Iwanaga et al., 2008; Qiu et al., 2010; Ritz et al., 2012; Waki et al., 2008). However, many of these genes were found to be altered prior to the development of hypertension.

4.1.2. Hypothesis

Hypertension will lead to alterations in gene expression in particular those related to vascular remodelling, endothelial signalling and inflammation.

4.1.3. Aims

The present study aimed to investigate whether hypertension leads to alterations in gene expression and functional pathways, with a particular interest in those related to structural alterations identified in the previous chapter such as,

collagen, endothelial signalling and inflammation and also any genes differentially expressed related to I3C.

4.2. Methods

4.2.1. Animals

Experiments were conducted on Cyp1a1 Ren2 transgenic rats processed as described in chapter 2.3. This studied examine gene expression in the same animals from the young 6-month cohort (control diet, no I3C, n=8) and hypertensive (0.15% I3C, n=8), which were used for the vascular protein levels experiments (Chapter 3). Briefly, these animals were run as part of the young 6-month cohort and were saline perfused, the brains snap frozen and then stored at -80˚C until they were processed for microarray analysis.

4.2.2. RNA extraction

RNA was extracted from a subcortical dissection (-3.14mm to -5.15mm from Bregma), inclusive of both grey and white matter regions (Paxinos and Watson, 1998)) using Trizol protocol according to manufacturer's instructions (Invitrogen, Paisley, UK), as covered in detail in chapter 2.3.3.

4.2.3. Microarray analysis

Microarray RNA was collected from 8 animals per group. Samples were hybridized to Affymetrix Genechip rat gene 1.1 array representing >27 000 genes, using Gene Titan multi-channel instrument carried out by Alison Downing as described in chapter 2.3.3.

Genomic data was analysed by myself with assistance from Alison Downing when needed and was analysed by ANOVA with significance set at $p<0.005$. Pathway analysis was carried out using Ingenuity software on differentially expressed genes at a significance of $p<0.01$.

4.3. Results

4.3.1. Hypertension induced significant alterations in gene pathways of cell morphology, development, nervous system development and function

Microarray analysis was undertaken in animals from the young 6-month cohort to investigate alterations in gene expression, in response to hypertensive induced vascular alterations. Of the 27 000 genes investigated, 48 were differentially expressed, with 23 upregulated and 25 downregulated (Table 4.1), in hypertensive animals compared to normotensive ($p<0.005$). To provide evidence of biological functions, pathway analysis was carried out on 109 genes, differentially expressed at a less conservative significance of $p<0.01$. There was evidence of alterations to 4 functional pathways, which were ranked by their significance (Figure 4.1). The most significant was pathway 1; cellular morphology, development, nervous system development and function (score 44, Figure 4.2). Followed by pathway 2; lipid metabolism, molecular transport, small molecules biochemistry (score 20, Appendix B.1), pathway 3; organ development and morphology (score 19, Appendix B.2) and pathway 4; cell morphology, function and maintenance (score 15, Appendix B.3). Overall, hypertension induced alterations in many growth factor genes; including Vascular endothelial growth factor (VEGF), Fibroblast growth factor (FGF) and

Insulin like growth factors (IGFs), and also Collagen IV, ion channels and inflammatory related genes.

Upregulated genes at $p<0.005$			
Gene	Assignment	p-value ($x10^3$)	Fold change (normotensive vs. hypertensive)
-	unannotated	0.249	1.103
-	unannotated	0.277	1.291
-	unannotated	0.315	1.155
-	unannotated	0.319	1.123
Sox12	SRY (sex determining region Y)- box 12	0.725	1.097
Tbc1d10a	TBC1 domain family, member 10a	0.918	1.154
Prss53	Protease, serine 53	1.391	1.112
Zbtb3	Zinc finger and BTB domain containing 3	1.429	1.136
-	Unannotated	1.912	1.200
Nfkbia	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor	1.977	1.384
Usp21	Ubiquitin specific peptidase 21	2.022	1.072
Prdm4	PR domain containing 4	2.127	1.056
Nmnat3	Nicotinamide nucleotide adenylytransferase 3	2.339	1.096
RGD1309036	Hypothetical LOC292874	2.419	1.158
-	Unannotated	2.600	1.110
Mgat5b	Mannosyl (alpha-1,6-)-glycoprotein beta-1,6-N-acetyl-glucosam	3.017	1.082
RGD1560703	Similar to coated vesicle membrane protein	3.100	1.114
RGD1566359	Similar to RIKEN cDNA B230219D22	3.159	1.168
Col4a2	Collagen, type IV, alpha 2	3.331	1.135
-	unannotated	3.660	1.130
-	unannotated	3.963	1.370

-	unannotated	4.053	1.205
RGD1560880	Similar to RIKEN cDNA 2310002J15	4.671	1.196
Downregulated genes at $p<0.005$			
Gene	Assignment	p-value ($x10^3$)	Fold change (normotensive vs. hypertensive)
Rpe65	Retinal pigment epithelium 65	0.335	-1.274
Cetn4	Centrin 4	0.545	-1.070
Ctsr	Cathepsin R	0.944	-1.152
Paip2	Poly (A) binding protein interacting protein 2	0.956	-1.093
Mast4	Microtubule associated serine/threonine kinase family	1.101	-1.172
Lifr	Leukemia inhibitory factor receptor alpha	1.146	-1.089
Ccdc59	Coiled-coil domain containing 59	1.561	-1.129
Adipor2	Adiponectin receptor 2	1.746	-1.168
Slc38a2	Solute carried family 38, member 2	1.924	-1.188
LOC683538	Similar to metaxin 3	1.965	-1.233
-	Unannotated	2.147	-1.112
Usp54	Ubiquitin specific peptidase 54	2.674	-1.192
Cdc42bpa	CDC42 binding protein kinase alpha	2.903	-1.111
LOC686547	Similar to TBC1 domain family member 4	3.114	-1.191
Sass6	Spindle assembly 6 homolog	3.222	-1.100
LOC100364673	Hypercoagulability related protein	3.248	-1.143
LOC687856	Similar to myeloid cell surface antigen CD33 precursors	3.272	-1.161
Hist1h2bb	Histone cluster 1	3.846	-1.137
-	Unannotated	3.935	-1.137
Mcc	Mutated in colorectal cancers	4.027	-1.163
Yipf5	YIP1 domain family, member 5	4.072	-1.082

-	Unannotated	4.184	-1.229
Gpr116	G- protein coupled receptor 116	4.341	-1.210
Hpgd	Hydroxyprostaglandin dehydrogenase 15	4.357	-1.175
-	Unannotated	4.910	-1.105

Table 4.1: Upregulated and Downregulated gene expression with hypertension

Details of the genes found to be upregulated and those downregulated in expression in hypertensive animals compared normotensive including gene assignment, significance and fold change.

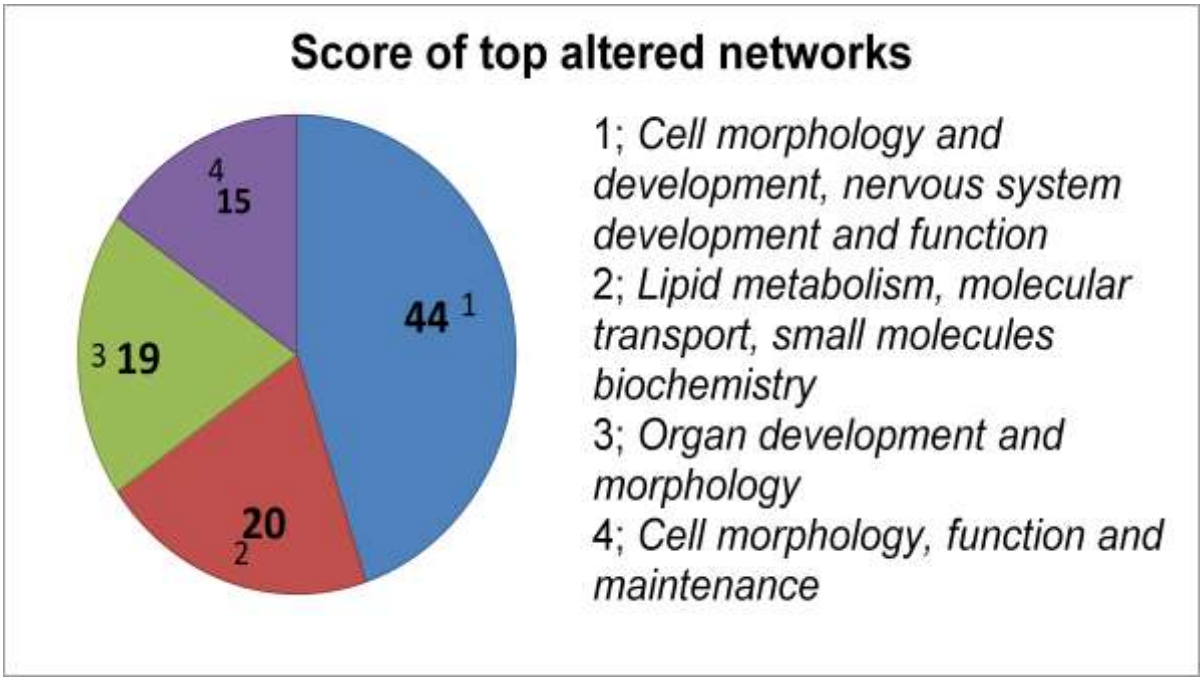

Figure 4.1: Pathway analysis of differential gene expression
Pathway analysis provided evidence of alterations within 4 different biological pathways. The most significantly altered gene pathway with hypertension is the pathway for cell morphology and development, nervous system development and function. Within this pathway there is differential gene expression of growth factors and ion channels in hypertensive animals when compared to normotensive.

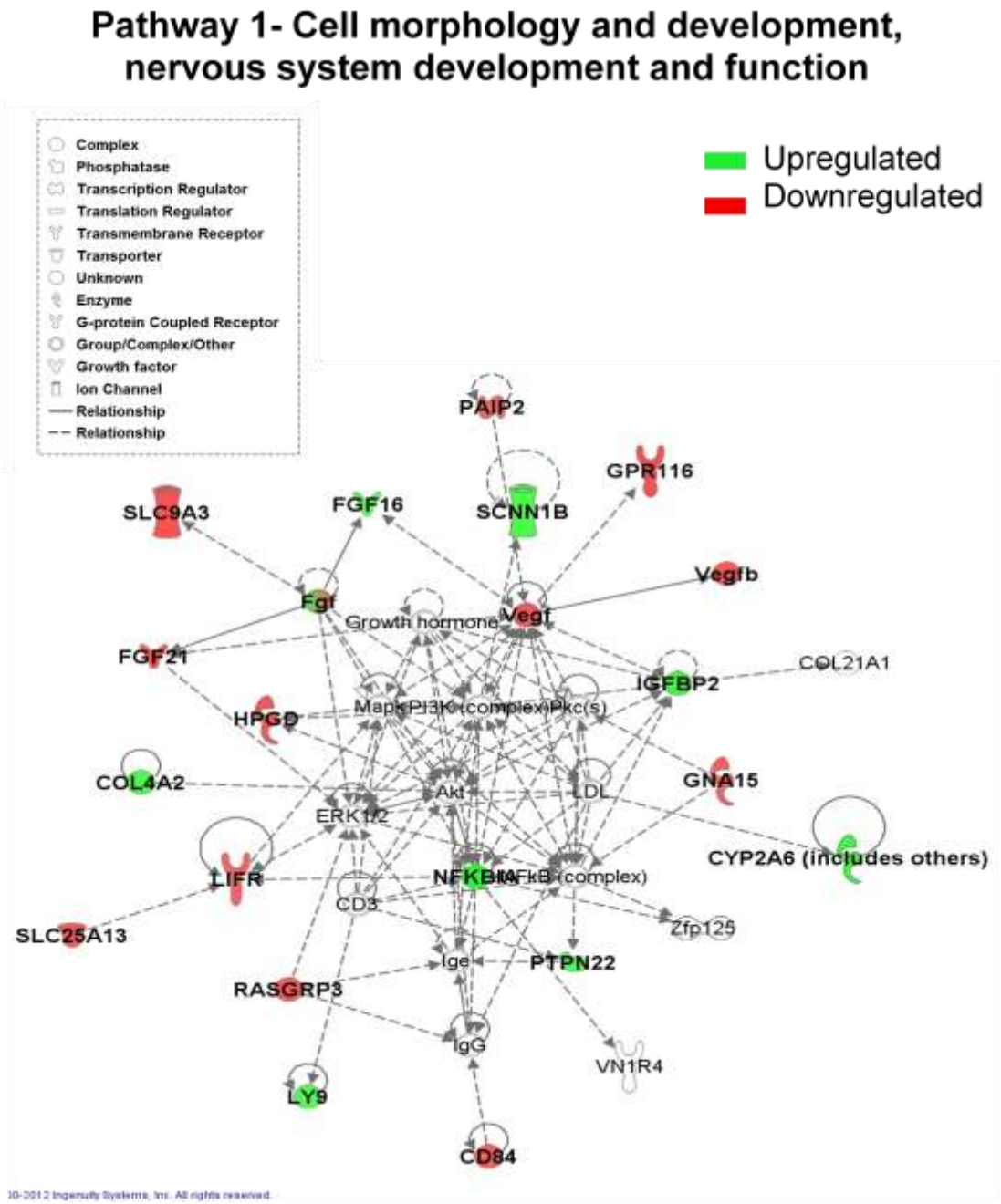

Figure 4.2: Pathway 1- Cell morphology and development, nervous system development and function

The most significantly altered pathway with hypertension. Upregulated genes are in green and downregulated genes are in red in hypertensive animas versus normotensive. Each gene is attributed a status symbol, with the key included within the figure. Within this pathway hypertension leads to differential gene expression of collagen, growth factors, ion channels, inflammatory and endothelial signalling.

4.4. Discussion

Within the hypertensive literature there is a lack of knowledge as to the genes that are altered as a result of hypertensive induced cerebrovascular pathology. The present study sought to investigate gene alterations in the young 6-month cohort, which had previously shown evidence of alterations to vascular structure, endothelial signalling and inflammatory response. The present study, consistent with immunohistochemical findings from the previous chapter, provided evidence of differential expression in collagen IV, eNOS signalling pathway and inflammatory genes. Crucially the present study also found evidence that hypertension caused differential expression of growth factor and ion channel genes, which had not been identified or examined in the previous study. Thus, the findings within the present study indicate that hypertension leads to alterations at the cellular gene expression level as well as the structural level. These findings provide a crucial insight into the mechanism of hypertensive cerebrovascular alterations.

4.4.1. Differential gene expression with hypertension

The present study identified a small number of genes differentially expressed with hypertension. This was not surprising, as although the previous study identified vascular alterations, these were relatively subtle. Additionally gene expression was investigated within a subcortical dissection, which unlike the previous chapter would have included white matter regions such as the internal capsule. The presence of a larger region may have diluted some of the gene changes observed. To provide mechanistic insight these genes were characterised into functional pathways, 4 of which were found to be significantly altered with hypertension when compared to normotensive animals. In general pathway analysis found that more genes were

downregulated with hypertension than upregulated, indicating that hypertension affects normal gene response in a negative manner, silencing gene expression. The functional pathways are discussed in the following sections.

4.4.1.1. Pathway 1- Cellular morphology and development, nervous system development and function

The present study found the most significantly altered pathway as a result of hypertension was: cellular morphology, development, nervous system development and function. Overall, this pathway provided evidence of differential gene expression of collagen IV, growth factors and eNOS signalling pathway.

4.4.1.2. Differential expression of collagen with hypertension

The present study found evidence of increased expression of the collagen 4A2 gene (COL4A2) and is in agreement with evidence of altered collagen IV expression in chapter 3 of this thesis. However, this finding is in disagreement with the SHR and SHRSP models, which have failed to provide evidence of altered COL4A2 gene expression at similar durations of hypertension (Iwanaga et al., 2008; Ritz et al., 2012), but alterations in collagen IV gene expression has also been found in humans, with small vessel disease (Dichgans and Hegele, 2007). Therefore, presenting a similarity between gene alterations found with hypertension in our model and those observed in humans with cerebrovascular and WM pathology. The increased collagen gene expression may indicate that the structural alterations found in the previous study will progress to basement membrane thickening. Thus, this finding both supports previous data found within this thesis and provides a mechanistic insight.

4.4.1.3. Differential expression of growth factors

A large population of genes differentially expressed with hypertension were found to be growth factor genes. Firstly, there was a decreased expression of vascular endothelial growth factor (VEGF) and VEGFb genes, in hypertensive animals when compared to normotensive. VEGFb gene has been found to bind to the FLT-1 VEGF receptor negatively regulating angiogenesis (Roberts et al., 2004). In addition within this pathway, VEGF is associated with a decreased expression of G-protein coupled receptor 116 (GPCR 116) gene and poly (a) binding protein interacting protein 2 (PAIP2). The function of GPCR 116 is not fully known but it has been shown to be localised on the vasculature (Wallgard et al., 2008). PAIP2 on the other hand is crucial for VEGF mRNA stabilisation and activation (Takahashi and Shibuya, 2005). These findings are in agreement with the SHR model, which has shown VEGF to be increased during the developmental stage of hypertension (Ritz et al., 2012) but decreased during established hypertension (Wang et al., 2004). These alterations in VEGF expression in the SHR models were also found to be associated with impaired angiogenesis (Wang et al., 2004). Similar findings have been observed in the SHRSP model with decreased VEGF mRNA expression (Jesmin et al., 2007). Functionally, VEGF is a signalling protein produced by cells to signal angiogenesis but it also plays a role in endothelial function, as VEGF forms part of the MAP-kinase pathway and can activate the expression of eNOS – promoting vasodilation (Lal et al., 2001; Servos et al., 1999). The present findings may indicate endothelial dysfunction due to a decreased expression of a key promoter of vasodilation coinciding with morphological findings of string-like vessels. Notwithstanding, the evidence of increased eNOS expression in the vasculature of the young 4-month hypertensive

animals, in vessels that did not exhibit signs of vascular remodelling represents alterations in the eNOS pathway as a result of hypertension.

Within this pathway there was also differential gene expression of fibroblast growth factor (FGF) genes, which can influence cell growth and differentiation and bind to the extracellular matrix through linkage with heperan sulphate. FGFs have also been found to induce activation of the MAP-kinase pathway and associated with NO dependant and independent angiogenesis, unlike VEGF (Bonneh-Barkay and Wiley, 2009). Within the present study there was increased gene expression of FGF 16 and a decreased gene expression of FGF 21. The specific role of both of these genes is not fully understood, but FGF 16 knockout studies have found it to be crucial in the formation of the cardiovascular system (Lu et al., 2008) and FGF 21 has been associated with alterations to energy metabolism (Coskun et al., 2008). It is difficult to directly compare these findings to those documented in the SHR models, but it has been shown that the SHRs have decreased expression of FGFs (Cuevas et al., 1996) and impaired energy metabolism (Ritz et al., 2012).

FGF21 has been characterised as playing a pivotal role in metabolic regulation of glucose and leptin metabolism (Cuevas-Ramos et al., 2009). As well as FGF 21, there was also an increased expression of Insulin growth factor binding protein (IGFBP), important for insulin energy metabolism. IGFBP regulates the expression of Insulin growth factor (IGF) in an inhibitory fashion and importantly IGF has been found to be decreased in expression in the aging brain (Trejo et al., 2004). Thus, the finding of increased IGFBP gene expression is suggestive of decreased IGF expression. This is in agreement with a study carried out in the SHRSP model, providing evidence of decrease IGF expression, accompanied with

decreased VEGF and eNOS expression (Yoshitomi et al., 2011). This is due to upregulation of IGF being stimulated by the expression of PI3K-ARK, members of the MAP-Kinase pathway, also responsible for VEGF and eNOS expression (Burgering and Coffer, 1995; Michell et al., 1999). Within the present study alterations in the expression of growth factors that are related to energy metabolism could represents a possible de-harmonisation between neurons and capillaries, with energy reserves not being met in the subcortical region of the brain. Interestingly, studies have investigated the role of IGFBP in relation to myelination and found that there is interplay between IGFBP and thus IGF expression and myelin integrity. Overexpression of IGFBP is linked with demyelination and increased neuronal apoptosis (D'Ercole et al., 2002; Doublier et al., 2000; Ni et al., 1997), whereas decreased expression of IGFBP is linked with hypermyelination (D'Ercole et al., 1996; D'Ercole et al., 2002; Dentremont et al., 1999; Ye et al., 1995). Following studies in this thesis will examine the expression of myelin with hypertension and investigate if there is any relationship between the gene expression, vascular alterations and myelin integrity.

Moreover, alterations in IGFBP expression may also relate to the vascular inflammation observed with hypertension in the previous study, as the IGF can be regulated by the expression of cytokines, reactive oxygen species and hemodynamic alterations (Delafontaine et al., 2004). IGF receptors have been found to be located on neurons and require insulin transport through the BBB. Interestingly, studies have also found IGF receptors on vascular smooth muscle and endothelial cells (King et al., 1985) and overexpression has been associated with aberrant vascular contractility (Zhao et al., 2001). During hypoxic-ischemia IGF has been found to be increased

soon after injury leading to an increased expression of IGFBP located on microglia and astrocytes (Beilharz et al., 1998). This indicates a possible mechanism between vascular remodelling, endothelial signalling alterations and inflammation, which could cause alterations to myelin integrity.

4.4.1.4. Differential inflammatory gene expression

Previous studies within this thesis found increased expression of microglial cells associated with the vasculature in hypertensive animals when compared to normotensive. Microglial cells are known to have both neuroprotective and degenerative actions. Gene alterations within this study also provides evidence of a protective inflammatory response with an increased expression of Nuclear factor of Kappa light polypeptide gene enhancer in B cells (NFκB) Inhibitor alpha (NFκBIA) gene with hypertension, found to be neuroprotective and increased in response to oxidative stress (Traenckner et al., 1994; Xu et al., 2002). NFκB is a transcription factor responsible for pathogen response, immune activation and cell survival. During inactivation, NFκB is anchored to the cytoplasm by inhibitors such as NFκBIA. Studies in ischemia have shown that the inhibition of NFκB by activation of NFκBIA protects the brain from injury (Treadwell and Singh, 2004; Xu et al., 2002). However, NFκB can be neuroprotective, preventing pro-apoptotic c- Jun N-terminal kinase (JNK), signalling through TNF receptors (De Smaele et al., 2001). NFκBIA is linked with downregulated hydroxyprostaglandin dehydrogenase-15 (HPGD), which is involved in the inactivation of prostaglandins and inflammatory cytokines (Otani et al., 2006; Tong et al., 2006a). Within the present study it is unknown whether increased NFκBIA gene expression is in response to inflammatory

mediators released by microglia or activated by another signalling mechanism but it does suggest a form of inflammatory response.

In addition, the present study also found downregulation of Leukemia inhibitory factor (LIHF) gene, known to be upregulated in the acute inflammatory phase response and can release both anti-inflammatory and pro-inflammatory mediators (Gadient and Patterson, 1999; Suzuki et al., 2005). LIHF can act on a broad range of cells and can be expressed on endothelial cells, regulating energy dependent astrocytic differentiation (Mi et al., 2001; Suzuki et al., 2005). Decreased LIHF gene expression has also been associated with demyelination (Butzkueven et al., 2002). In turn, associated with the LIHF gene, the SLC25A13 gene known as Centrin was downregulated. Centrin is a mitochondrial aspartate-glutamate carrier, expressed in subcortical neurons of the brain and a major component of the malate aspartate shuttle pathway transferring reducing agents from NADH to mitochondria. Within this study there is a decrease in the Centrin gene expression, which may impair energy formation in neurons (Contreras et al., 2010). Overall, these findings further indicate that hypertension induces alterations to the energy metabolism associated with altered inflammatory gene expression.

Another inflammatory marker which is downregulated with hypertension in this study is the chemokine CD84 involved in cellular and signal transduction and associated with the acute phase of inflammatory response (De la Fuente et al., 1997). Within this pathway CD84 is associated to RAC-alpha serine/threonine-protein kinase (AKT) part of the eNOS activation pathway. The downregulation of CD84 is likely due to the loss of eNOS expression with increased duration of hypertension. However, it could be postulated that CD84 was upregulated initially with

hypertension as eNOS is increased in the young 4-month cohort. There is also downregulation of other genes associated with the ARK pathway. RASGRP3 gene encodes for guanine nucleotide exchange factor which activates small GTPases. RASGRP3 has a calcium binding EF hand motif and interacts with Protein kinase C (Lorenzo et al., 2001). Although the role of this gene is not fully understood, in the human brain RASGRP3 has been shown not only to be associated with the ARK pathways but can directly activate eNOS expression (Lorenzo et al., 2001).

As discussed previously a limitation of the vascular study carried out in chapter 3 was that microglia was the only inflammatory cell measured. In the present study there is evidence of upregulation of the inflammatory cells- leukocytes with hypertension, as there is upregulation of Protein tyrosine phosphatase 22 (PTP N22), a regulator of the immune system and the expression of T lymphocytes (Kyogoku et al., 2004). There is also an increased expression of lymphocyte antigen 9 (LY9) with hypertension, a receptor on T and B lymphocytes and has been shown to be activated in the mouse brain in response to hypoxic ischemia insults (De la Fuente et al., 2001; Hedtjarn et al., 2004). Increased expression of leukocytes has been found in acute severe surgically produce hypertension in the transverse aortic coarctation model (Poulet et al., 2005) and the malignant hypertensive SHRSP model with occurrence of vascular remodelling (Kimoto-Kinoshita et al., 1999). In the SHRSP model increased leukocyte expression was associated with decreased NO (Kimoto-Kinoshita et al., 1999). Studies in the SHR model have also provided evidence that leukocytes can express angiotensinogen, which can contribute to vascular remodelling (Takemori et al., 2000). In addition, it has also been found that in Angiotensin II and DOCA-salt animals models of hypertension, generated to lack T-

lymphocytes fail to become hypertensive (Guzik et al., 2007) and that vascular inflammation can be stimulated by other mechanism not exclusively due to Angiotensin II overexpression (Marvar et al., 2010). Therefore, the findings within this study provide evidence of generalised inflammatory response with hypertension, which can be associated with alterations in endothelial signalling.

4.4.1.5. Differential gene expression of genes related to ion and small molecule transport

Ion channels are crucial for the trafficking of ions across cell membranes, alterations in the concentration of ions can lead to alterations in brain function through changes in membrane potential and action potential firing. Within the present study hypertension induced alterations to ion channel gene expression. The sodium channel, non voltage-gated 1 beta (SCNN1B), was found to be upregulated. SCNN1B is amiloride sensitive, located on the epithelium and also found to be increased in patients with Liddle's syndrome - a genetic form of hypertension (Shimkets et al., 1994; Tong et al., 2006b). Studies in experimental models of hypertension have investigated the role of gene polymorphisms and the predisposition for the development of hypertension but have not found significant results (Grunder et al., 1997). Peripheral studies have found that this gene and several others of similar function are activated in the kidney by increased aldosterone expression and correlate with increased blood pressure (Jin et al., 2010). The SCNN1B gene has been shown to be expressed in the rat brain and is associated with the regulation of cerebrospinal fluid, interstitial sodium concentration and neuronal excitation (Amin et al., 2005). Alterations in CSF sodium concentrations, which exacerbate hypertension in the SHR model have been found after increased dietary

salt and were attributed to altered activity of Na^+ channels within the brain (Wang et al., 2010).

Another ion channel found to be altered with hypertension in this study was the SLC9A3 gene, which was found to be downregulated. This gene is located within chromosome 1 coding the Na+/H+ exchanger 3 and has been associated with blood pressure control in the SHR model (Szpirer et al., 1997) and hypertensive individuals (Zhu et al., 2004). Since ion channels can be located on endothelial cells, it could be postulated that the alterations in BBB tight junction expression shown in the previous study could also be associated with alterations to ion channels, allowing alterations in ion concentrations within the brain, which could signal to recruit microglia.

4.4.1.6. Metabolism of Indole-3-carbinol

Lastly, the CYP1a1 Ren2 rat model used within this thesis induces hypertension through dietary addition of I3C, allowing increased expression of mouse Ren2 gene coupled to CYP1a1 promoter. Microarray analysis yielded increased gene expression of CYP2a6, which is involved in the metabolism of aryl hydrocarbons and may been increased due to increased I3C (Fujii-Kuriyama et al., 1995; Loub et al., 1975). CYP2a6 also plays a role in the metabolism of Coumarin and nicotine (Rao et al., 2000). However, the application of I3C in this model leads to increased hepatic expression of Angiotensin II. It is not fully elucidated whether Angiotensin II can cross the BBB but a study by Paton et al., suggested Angiotensin II can enter the brain through the circumventricular organs and signal to the endothelium to release various vasoactive mediators including eNOS (Paton et al., 2008; Paton et al., 2007b). Since the present study did not identify Angiotensin II or

related gene expression with hypertension it is suggestive that the vascular alterations are not exclusive to Angiotensin II expression.

All in all, the gene changes observed with hypertension in pathway 1 indicate that there are model specific alterations, coupled with hypertensive induced alterations in collagen, growth factors, energy metabolism, inflammatory and ion channel gene expression. The pathway analysis also yielded a further 3 pathways differentially expressed with hypertension. However, the remainder of genes that were altered formed the following functional pathways - pathway 2 concerning lipid metabolism, molecular transport, small molecule biochemistry, followed by pathway 3 concerning organ development and morphology and pathway 4 concerning cell morphology, cellular function. These pathways contained a small number of differentially expressed genes that had similar functional characteristics as those altered in pathway 1. Thus, pathway 1 being the most significant and including several differentially expressed genes was discussed in full and the genes altered within the remaining pathways can be found within Appendix C.

4.4.1.7. Summary

The present study provided evidence of genes altered with hypertension, at a time point when initial structural alterations to the cerebrovascular had occurred and is in agreement with previous findings in the SHR literature showing differential expression in genes related to energy metabolism, ion channels and inflammatory markers (Zhou et al., 2005). However, the present study did not find any differential expression of soluble epoxide hydrolase (sEH) identified as a novel gene altered with hypertension in the SHR model. This may be due to sEH being identified from hypothalamic tissue at a different severity of hypertension. In turn, the severity of

hypertension could also explain for the relatively small amount of genes altered in this model.

A limitation of the present study is that it chose to examine gene expression within the whole region rather than using a vascular specific sample. Examining gene expression from a vascular enriched sample may have allowed for amplification of genes altered in response to hypertension. However, extracting vessels from the brain may have impaired the integrity of RNA, impairing the overall sample integrity and a larger volume of tissue would have been required to allow for sufficient sample to carry out microarray analysis. Thus, the present study decided to analyse gene expression using subcortical tissue, detecting gene expression within the whole region that had previously been found to be susceptible to hypertension and providing a global region specific assessment of the underlying mechanisms. Additionally this study did not carry out any post hoc qPCR assessments of the gene alterations found within the microarray. The rational was that firstly the alterations found within the microarray were relatively subtle and the gene alterations found were not overtly different from those morphological alterations observed within the previous chapter. It would have undoubtedly been useful to carry out follow up qPCR examination but both financial and time limitations need to be taken into account and the results found from this characterisation microarray are a useful tool for more in depth future studies, in which the severity of hypertension could be increased in both duration and level to yield more overt gene alterations.

4.4.1.8. Conclusion

The present study indicated that hypertension leads to alterations in gene expression. The differentially expressed genes are in agreement with findings in the previous chapter showing alterations in collagen, endothelial signalling and inflammatory gene expression. Novel mechanistic insight was provided with the findings that hypertension also leads to altered expression of growth factor and ion channel genes. In particular the identification of differential expression of IGFBP gene may indicate alterations in myelin integrity. The following study will build on the findings to investigate whether hypertension also leads to structural alterations in the WM in the young and aged brain.

5. The effect of hypertension on white matter integrity in the young and aged brain

5.1. Introduction

White matter occupies over half of the human brain (Fields, 2008) and is crucial for effective communication between brain regions, facilitating the flow of electrical signals over long distances (Arai and Lo, 2009; Bartzokis, 2004). However, with age WML are frequently observed, with around 50% of individuals over the age of 65 found to have WML (Enzinger et al., 2006), which are associated with age related cognitive decline (Van Swieten et al., 1991). Age aside, hypertension has been associated as the number one risk factor for the formation and exacerbation of WML (Pantoni and Garcia, 1995). The mechanisms that associate hypertension with the formation of WML in humans, are difficult to establish due to the coexistence of hypertension with other vascular risk factors such as diabetes (Epstein and Sowers, 1992; Mellitus, 2006). It is postulated that hypertension exerts deleterious effects to the cerebrovasculature, which promote alterations to the structural integrity of the brains more vulnerable regions, the WM (Pantoni and Garcia, 1995). Building on previous work within this thesis, this study sought to determine whether sustained hypertension may cause pathological alterations to the brains WM in the inducible model.

5.1.2. Hypothesis

Hypertension will lead to alterations to the integrity of white matter in the young and aged brain.

5.1.3. Aims

The study described in this chapter aimed to investigate the effects of hypertension on white matter integrity of the brain using the Cyp1a1 Ren2 rat model. White matter integrity was investigated in the young and aged brain. A detailed examination of myelin, oligodendrocytes, axons and microglial response was carried out and in the same animals it was determined whether there was any evidence of ischemic neuronal damage.

5.2. Methods

5.2.1. Animals

Experiments were conducted on the same Cyp1a1 Ren2 transgenic rats used for vascular immunohistochemical assessment described in chapter 2.1.2**.** Experiments were conducted on animals from the young 4-month, young 6-month and aged 4-month cohorts.

5.2.2. Histology and immunohistochemistry

Pathological assessments were carried out on paraffin embedded brain tissue sections (6µm) from both the young and aged cohorts as described in chapter 2.2. Sections were used for immunohistochemical assessment to investigate the integrity of white matter in the corpus callosum, fimbria, internal capsule and subcortical thalamic region of the brain. Adjacent sections were stained with H&E to determine ischemic damage to neuronal perikarya and overall structural integrity of the tissue.

5.2.3. Statistical analysis

Data from MBP intensity values, CC1 positive cell counts and number of microglia were analysed using unpaired t-tests. APP data was analysed by Fisher's exact test. Data for MBP and microglia activation is presented as a percentage of normotensive, although the raw average per hemisphere was statistically analysed. Significance was set at $p<0.02$. Each cohort was analysed separately and was not compared statistically

.

5.3. Results

5.3.1. Hypertension induced alterations to myelin basic protein in the young but not the aged brain

MBP immunohistochemistry was used as a marker of myelin integrity in normotensive and hypertensive animals. Each cohort was run, stained and analysed distinct to the other. The integrity of myelin was examined by measuring the intensity of MBP staining in the corpus callosum, fimbria, internal capsule and subcortical thalamic region. MBP was found to stain myelinated fibres and in general the distribution of staining was similar between all animals in the corpus callosum, fimbria and internal capsule. However, it was noted that there were variations in staining within the subcortex.

It was found that there was no significant difference in intensity of MBP in hypertensive animals from the young 4-month cohort, in the corpus callosum ($|t|= 1.34$, $df= 27$, $p=0.19$; Figure 5.1E, Table 5.1), fimbria ($|t|= 1.13$, $df= 27$, $p=0.27$; Figure 5.1F, Table 5.1) and internal capsule ($|t|= 1.13$, $df= 27$, $p=0.27$; Figure 5.1G, Table 5.1), when compared to normotensive animals. However, the intensity of subcortical thalamic MBP was found to be significantly increased in hypertensive animals when compared to normotensive ($|t|= 2.60$, $df= 27$, $p=0.01$; Figure 5.1H, Table 5.1). These results were also validated within this cohort by MRI analyses (Appendix E.1).

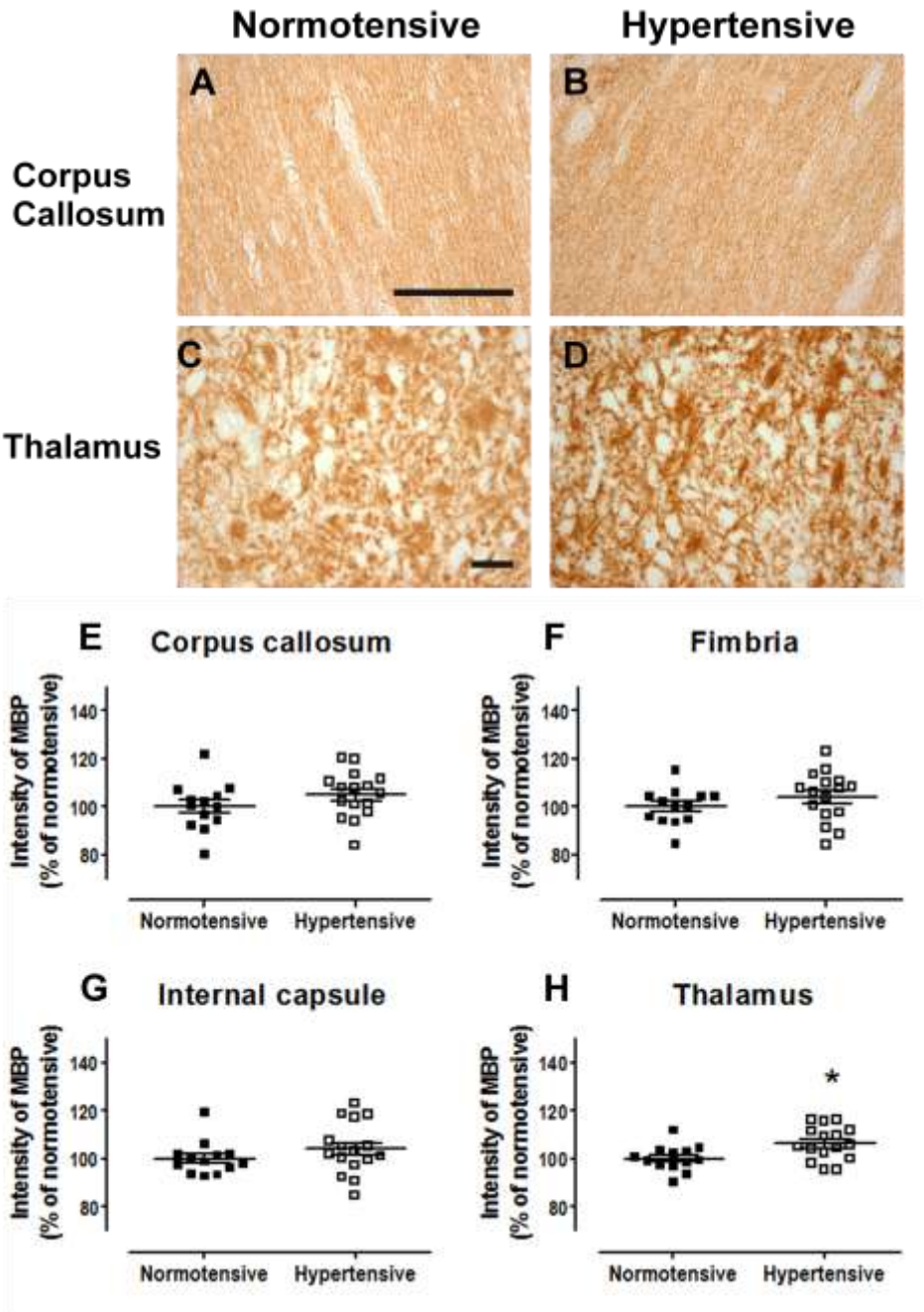

Figure 5.1: Increased subcortical thalamic MBP in the young 4-month cohort
Representative images of myelin basic protein (MBP) staining in the white and grey matter. In the corpus callosum MBP staining was not significantly different in normotensive animals (A, E) compared to hypertensive (B, E). The same finding was observed in other white matter tracts investigated; (F) fimbria and (G) internal capsule. In the subcortical thalamic region MBP staining was significantly increased in hypertensive animals (D, H) when compared to normotensive (C,H). Scale bar = 50µm. Graphs show mean ± SEM. Significance * p = 0.02 vs. Normotensive.

MBP data for the Young 4-month cohort							
Intensity per region							
Corpus Callosum		Fimbria		Internal Capsule		Thalamus	
Normotensive	Hypertensive	Normotensive	Hypertensive	Normotensive	Hypertensive	Normotensive	Hypertensive
0.34	0.46	0.40	0.36	0.42	0.41	0.36	0.37
0.41	0.45	0.50	0.46	0.54	0.47	0.44	0.43
0.51	0.46	0.43	0.45	0.46	0.45	0.38	0.41
0.43	0.51	0.46	0.47	0.48	0.53	0.39	0.43
0.40	0.41	0.41	0.50	0.42	0.53	0.38	0.43
0.43	0.42	0.45	0.43	0.44	0.46	0.35	0.38
0.42	0.48	0.41	0.41	0.45	0.47	0.40	0.41
0.38	0.43	0.36	0.47	0.42	0.45	0.40	0.45
0.39	0.45	0.45	0.49	0.46	0.46	0.41	0.45
0.45	0.40	0.45	0.46	0.44	0.54	0.38	0.42
0.42	0.40	0.43	0.39	0.44	0.38	0.39	0.39
0.45	0.50	0.44	0.38	0.46	0.42	0.39	0.37
0.44	0.43	0.40	0.53	0.45	0.56	0.40	0.45
	0.44		0.45		0.49		0.40
	0.47		0.42		0.44		0.40
	0.35		0.46		0.47		0.41

Table 5.1: MBP intensity values for each region from the young 4-month cohort

The above data represents the raw intensity values of MBP calculated for each region analysed for animals from the young 4-month cohort.

Similarly in the young 6-month cohort there was no significant difference in the intensity of MBP staining in the corpus callosum (|t|= 1.01, df= 27, p=0.32; Figure 5.2E, Table 5.2), fimbria (|t|= 0.92, df= 27, p=0.38; Figure 5.2F, Table 5.2) or internal capsule (|t|= 0.05, df= 27, p=0.96; Figure 5.2G, Table 5.2) of hypertensive versus normotensive animals. In contrast to the young 4-month cohort, the intensity of MBP was significantly decreased in the subcortical thalamic region of hypertensive animals compared to normotensive (|t|= 3.3, df= 21, p<0.01; Figure 5.2H, Table 5.2).

In the aged 4-month cohort there was no significant difference in the intensity of MBP between normotensive and hypertensive animals in the corpus callosum (|t|= 0.55, df= 18, p=0.59; Figure 5.3E, Table 5.3), fimbria (|t|= 0.17, df= 18, p=0.86; Figure 5.3F, Table 5.3), internal capsule (|t|= 0.61, df= 18, p=0.77; Figure 5.3G, Table 5.3) or subcortical thalamic region (|t|= 0.29, df= 18, p=0.76; Figure 5.3H, Table 5.3).

5.3.2. Hypertension does not induce alterations to the number of oligodendrocytes

To analyse whether myelin alterations may be due to underlying alterations in the oligodendrocyte cell population, the number of mature oligodendrocytes immunopositive for CC1 were counted within the subcortical thalamic region. CC1 stained oligodendrocyte cell bodies and the expression was found to be similar in all animals. There was no significant difference in the number of oligodendrocytes in hypertensive animals when compared to normotensive in young 4-month cohort (|t|= 0.70, df= 27, p=0.49; Figure 5.4G, Table 5.4), young 6-month cohort (|t|= 0.29, df=

21, p=0.77; Figure 5.4H, Table 5.4) and the aged 4-month cohort (|t|= 0.28, df= 18, p=0.78; Figure 5.4I, Table 5.4), each of which were analysed as individual cohorts.

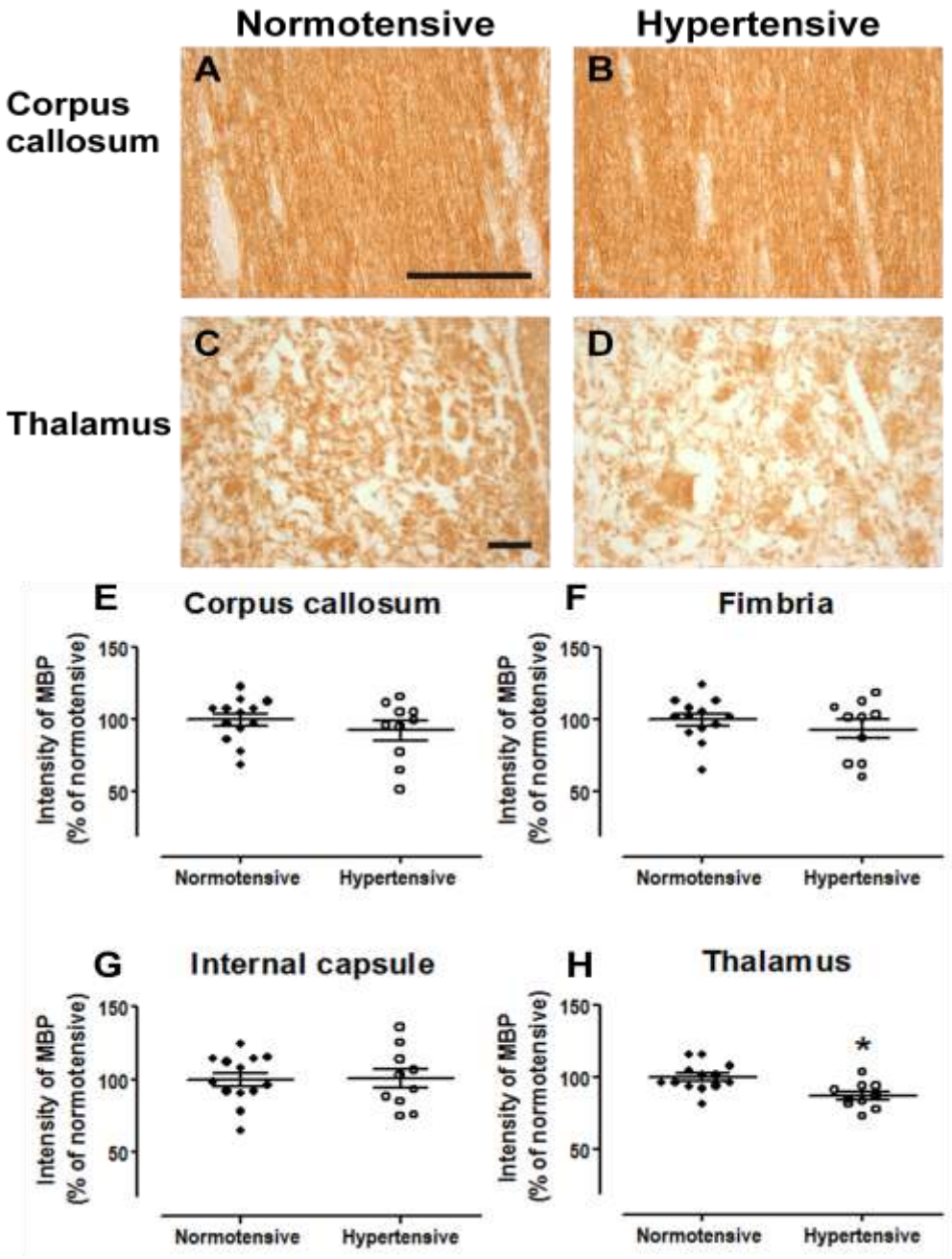

Figure 5.2: Decreased subcortical thalamic MBP in the young 6-month cohort
Images represent myelin basic protein (MBP) in the white and grey matter. In the corpus callosum MBP staining was not significantly different in normotensive animals (A, E) compared to hypertensive (B, E). The same finding was observed in other white matter tracts investigated; (F) fimbria and (G) internal capsule. In the subcortical thalamic region MBP staining was significantly decreased in hypertensive animals (D, H) when compared to normotensive (C,H). Scale bar = 50µm. Graphs show mean ± SEM. Significance * = $p \leq 0.02$ vs. Normotensive.

MBP data for the Young 6-month cohort							
Intensity per region							
Corpus Callosum		Fimbria		Internal Capsule		Thalamus	
Normotensive	Hypertensive	Normotensive	Normotensive	Normotensive	Hypertensive	Normotensive	Hypertensive
0.14	0.11	0.13	0.11	0.15	0.12	0.12	0.10
0.20	0.17	0.17	0.17	0.17	0.17	0.15	0.11
0.15	0.18	0.17	0.11	0.15	0.15	0.13	0.11
0.19	0.14	0.14	0.13	0.10	0.22	0.13	0.12
0.19	0.20	0.19	0.18	0.18	0.17	0.15	0.13
0.19	0.18	0.15	0.16	0.16	0.18	0.13	0.10
0.20	0.17	0.16	0.16	0.19	0.14	0.13	0.11
0.17	0.09	0.16	0.09	0.16	0.12	0.12	0.11
0.21	0.17	0.17	0.17	0.15	0.14	0.12	0.12
0.18		0.16		0.18		0.14	
0.16		0.16		0.18		0.14	
0.17		0.14		0.20		0.13	
0.12		0.10		0.13		0.11	

Table 5.2: MBP intensity values for each region from the young 6-month cohort

The above data represents the raw intensity values of MBP calculated for each region analysed for animals from the young 6-month cohort

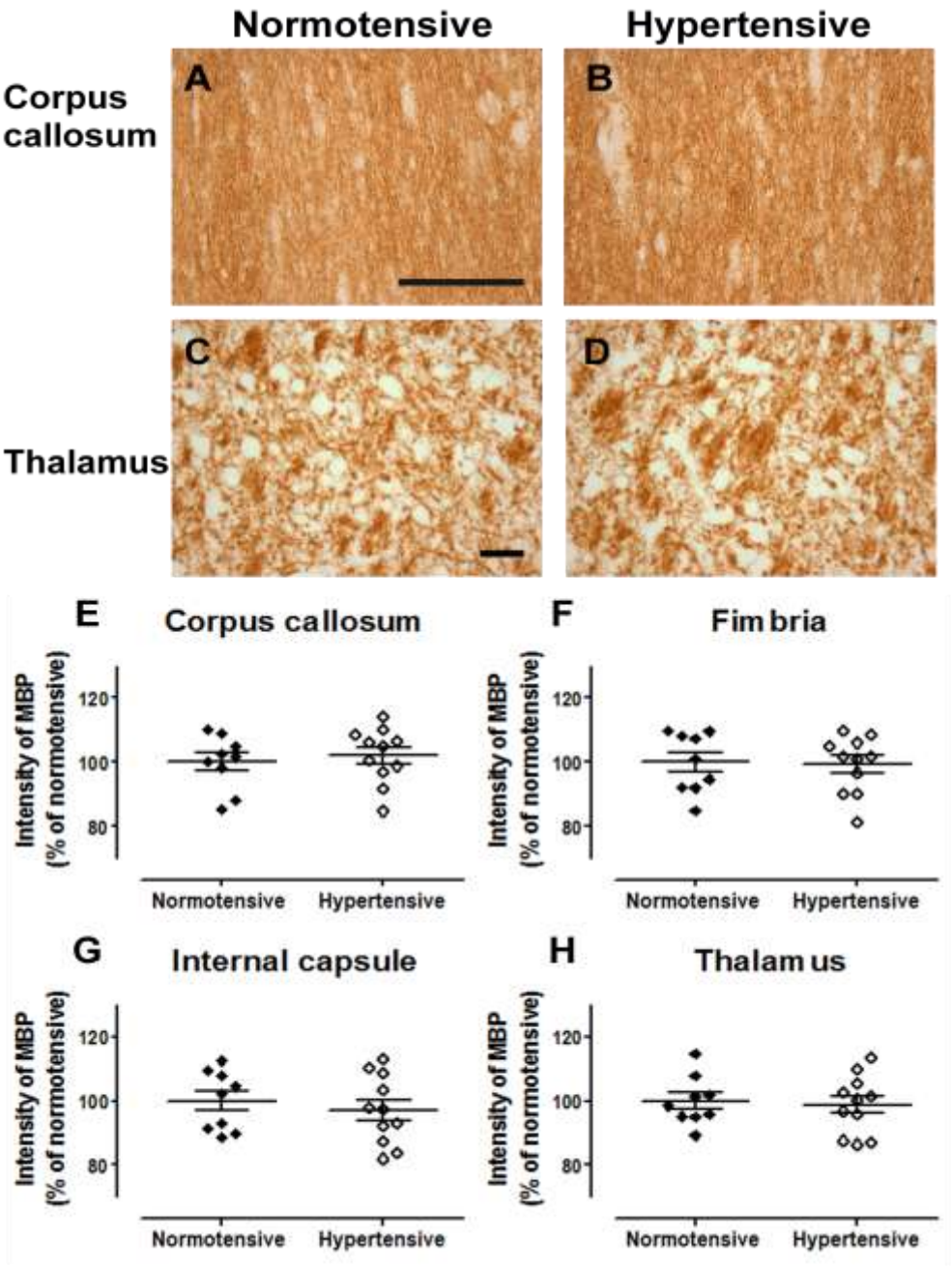

Figure 5.3: No alterations in MBP staining in the aged 4-month cohort

Representative images of myelin basic protein (MBP) in the white and grey matter tracts, visualized using an antibody specific for myelin basic protein (MBP). In the corpus callosum MBP staining was not significantly different in normotensive animals (A, E) compared to hypertensive (B, E). The same finding was observed in other white matter tracts investigated; (F) fimbria and (G) internal capsule. In the subcortical thalamic region MBP staining was not significantly different in normotensive animals (C,H) when compared to hypertensive (D, H). Scale bar = 50µm. Graphs show mean ± SEM. Significance * = $p \leq 0.02$ vs. Normotensive.

MBP data for the Aged 4-month cohort							
Intensity per region							
Corpus Callosum		Fimbria		Internal Capsule		Thalamus	
Normotensive	Hypertensive	Normotensive	Hypertensive	Normotensive	Hypertensive	Normotensive	Hypertensive
0.63	0.54	0.30	0.26	0.37	0.28	0.20	0.20
0.67	0.70	0.33	0.34	0.32	0.33	0.24	0.22
0.56	0.59	0.28	0.31	0.30	0.33	0.19	0.18
0.55	0.68	0.31	0.35	0.35	0.38	0.20	0.22
0.64	0.64	0.35	0.33	0.38	0.37	0.21	0.18
0.70	0.68	0.36	0.34	0.35	0.37	0.23	0.23
0.65	0.63	0.36	0.29	0.37	0.28	0.21	0.20
0.66	0.73	0.30	0.36	0.30	0.35	0.21	0.21
0.71	0.71	0.35	0.33	0.31	0.31	0.20	0.24
	0.62		0.33		0.32		0.21
	0.67		0.29		0.30		0.18

Table 5.3: MBP intensity values for each region from the aged 4-month cohort

The above data represent the raw intensity values of MBP calculated for each region analysed for animals from the aged 4-month cohort

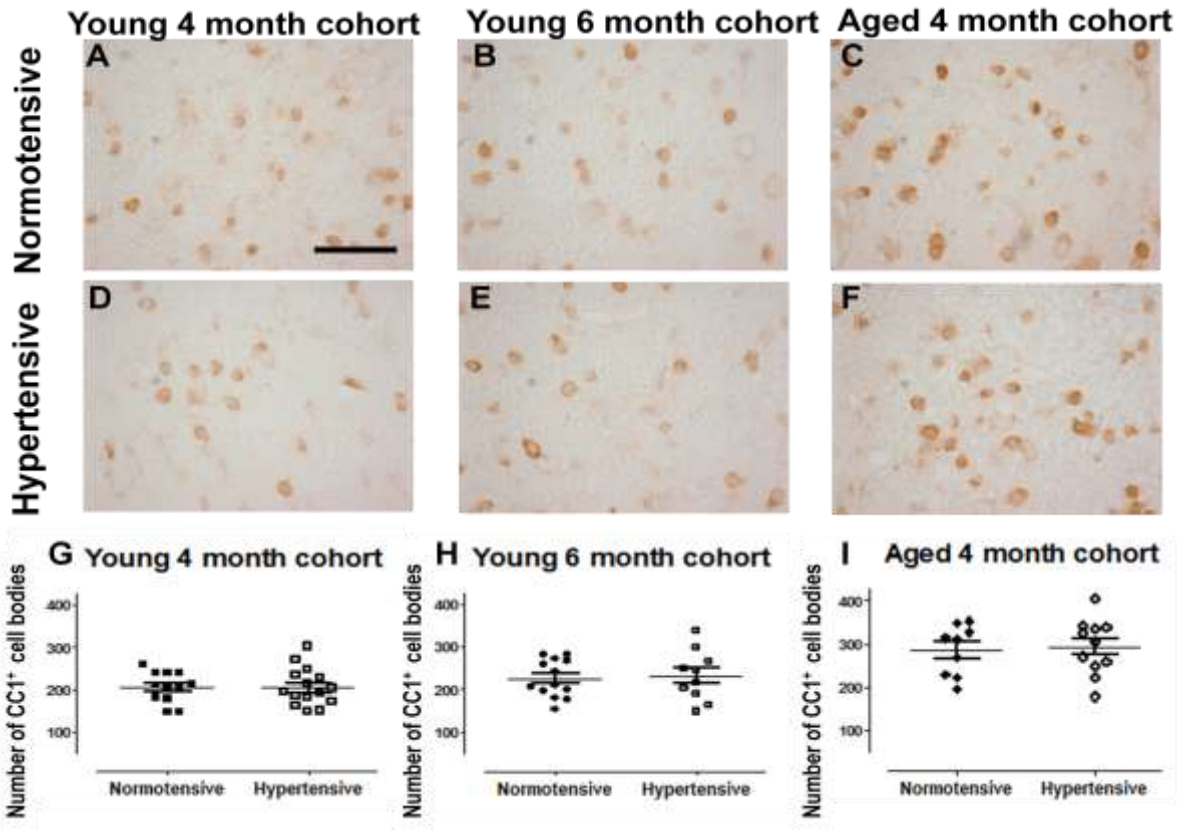

Figure 5.4: No change in the number of oligodendrocytes within the subcortical thalamic region

Representative images of mature oligodendrocytes visualized by staining with antibody specific for $CC1^+$ in the subcortical thalamic region in (A, D) young 4-month, (B E) young 6-month and (C, F) aged 4-month cohort. There were no significant differences in the number of $CC1^+$ oligodendrocytes in hypertensive animals (D, E, F) versus normotensive (A, B, C). Scale bar =50µm. Graphs show mean ± SEM.

Number of $CC1^+$ oligodendrocytes within the subcortex of each cohort					
Young 4-month cohort		Young 6-month cohort		Aged 4- month cohort	
Normotensive	Hypertensive	Normotensive	Hypertensive	Normotensive	Hypertensive
241.0	184.5	201.0	203.5	196.0	178.5
207.5	160.5	176.5	163.5	223.5	250.0
147.0	180.0	283.0	298.5	230.0	325.0
241.0	269.5	196.0	265.5	314.0	223.0
211.5	149.0	154.0	245.5	269.5	305.5
240.0	193.5	180.5	250.0	327.0	259.0
179.0	170.0	210.5	189.0	310.5	405.0
259.5	190.0	205.5	337.5	349.0	337.5
182.5	148.5	258.0	149.0	353.0	270.5
148.5	212.0	268.0			335.0
214.5	202.0	273.0			341.5
206.0	301.0	283.0			
	232.5	242.5			
	245.5				
	226.0				
	207.0				

Table 5.4: Number of $CC1^+$ oligodendrocytes within the subcortex

The above table represents the raw number of $CC1^+$ oligodendrocytes within the subcortex for each of the cohort analysed. Although the cohorts are presented together they were analysed separately.

5.3.3. Hypertension induced minimal axonal pathology

APP is an axonal transport protein and is normally localised to the cytoplasm of neurons, but when there is axonal disruption APP becomes accumulated at the site of injury. Axonal pathology was examined by the presence or absence of APP positive axonal bulbs. There was no evidence of axonal bulbs in the young 4-month cohort (Figure 5.5 A-D). In 6 out of 9 animals analysed from the young 6-month cohort, there was no evidence of axonal bulbs, but in the remaining 3 out of 9 hypertensive animals small areas of axonal bulbs were observed in the subcortical thalamic region (Figure 5.5 E,F).

Overall there was no significant difference in presence of axonal bulbs between normotensive and hypertensive animals (p=0.05) from the young 6-month cohort. In the aged 4-month cohort small patches of axonal bulbs were observed within the subcortical thalamic region in 6 out of 9 normotensive and 9 out of 11 hypertensive animals, with no significant difference between normotensive and hypertensive animals (p=0.62.,Figure 5.5 G,H).

5.3.4. Hypertension induced no overt damage to neuronal perikarya in the young and aged cohorts

H&E staining was used to determine the presence of ischemic neuronal perikarya and overall structural integrity of the brain. WM regions were investigated for the presence or absence of tissue pallor, microbleeds and haemorrhages. The hippocampus, fimbria and subcortical thalamic regions were investigated for the presence or absence of ischemic damage to the neuronal

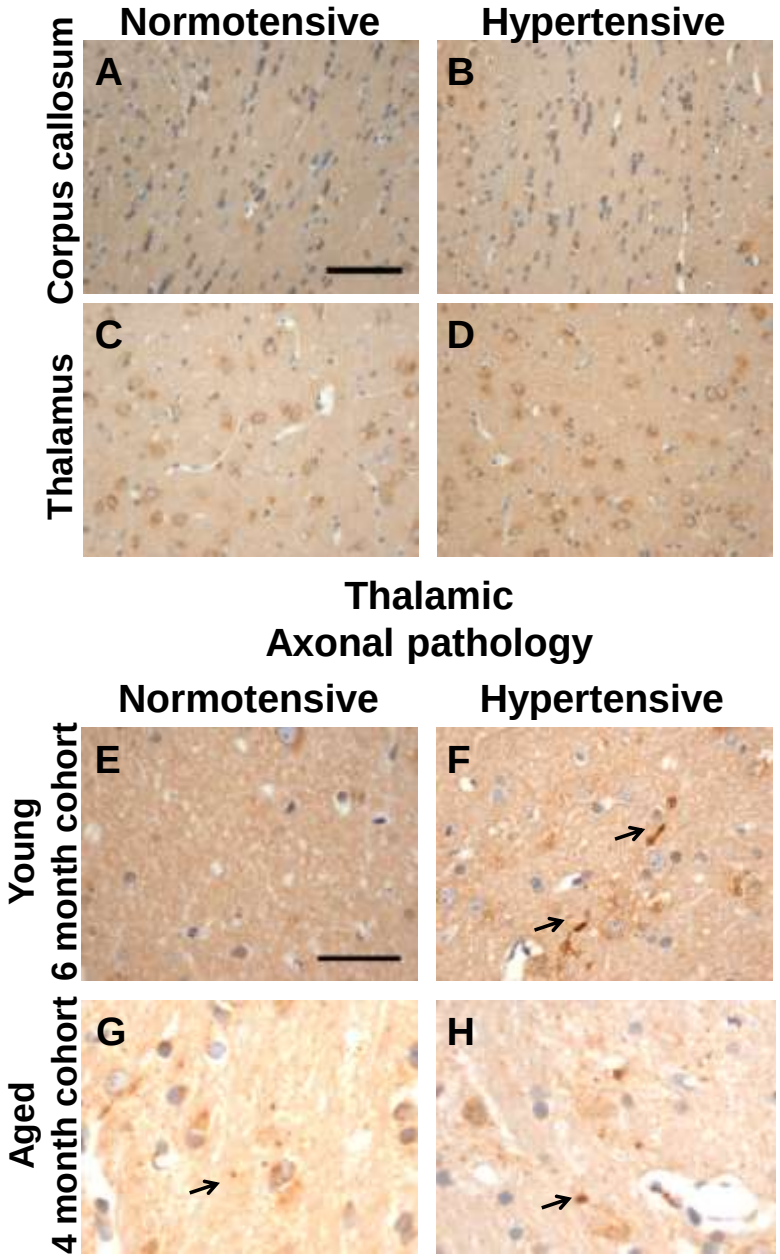

Figure 5.5: Minimal axonal pathology

Axonal pathology was assessed by the presence of axonal bulbs, visualised by aggregation of antibody specific for amyliod precursor protein (APP) (arrows). In the majority of white and grey matter regions there was no evidence of axonal bulbs in each cohort with none found in either normotensive (A) or hypertensive animals (B) in the young 4-month cohort. Similar findings were observed in animals from the young 6-month cohort. However, in a subset of hypertensive (D) animals in the young 6-month cohort there were small areas of subcortical thalamic axonal pathology, which was not observed in any normotensive animals (C). In the aged 4-month cohort small areas of the subcortical thalamic region contained axonal bulbs, which were found in normotensive (E) and hypertensive (F) animals with no significant difference between them. Scale bar =100µm.

perikarya, tissue pallor, microbleeds and haemorrhages. Changes to ischemic neurons were identified by the appearance of eosinophilic cytoplasm and pyknotic nuclei (Jortner, 2006). There was no evidence of damaged neurons or overt structural pathology in the young 4-month cohort (Figure 5.6). In the majority of animals from the young 6-month cohort there was no evidence of neuronal damage or overt structural pathology (Figure 5.6). However, one hypertensive animal had evidence of small microbleeds within the corpus callosum, with pallor to the surrounding tissue (Figure 5.6E). In the aged 4-month cohort, the majority of animals had no evidence of neuronal damage, except one hypertensive animal that had evidence of hippocampal neuronal damage, represented by eosinophilic (pink) staining around the cell and was also observed to be associated with a region of vaculation (Figure 5.6F). No overt structural pathology was observed with H&E in the corpus callosum, hippocampus, fimbria, internal capsule or the subcortical thalamic region in either hemisphere at any time point.

Previous studies in the inducible hypertensive model described gross morphological alterations within the kidneys of hypertensive animals. As this study found evidence of minimal alterations to the structural integrity of the brain, the kidneys of animals from the young 4 and 6 month cohort were qualitatively examined for evidence of gross morphological alterations. Previous studies had found that the kidneys of hypertensive animals had vascular enlargement, tubule dilation, accumulation of inflammatory cells and protein accumulation or necrosis of the glomeruli in I3C induced animals (Graciano et al., 2007) and these finding were replicated in hypertensive animals from the present study (Figure 5.7). Indicating that

hypertension is exerting deleterious effects to the periphery which are far less severe in the brain.

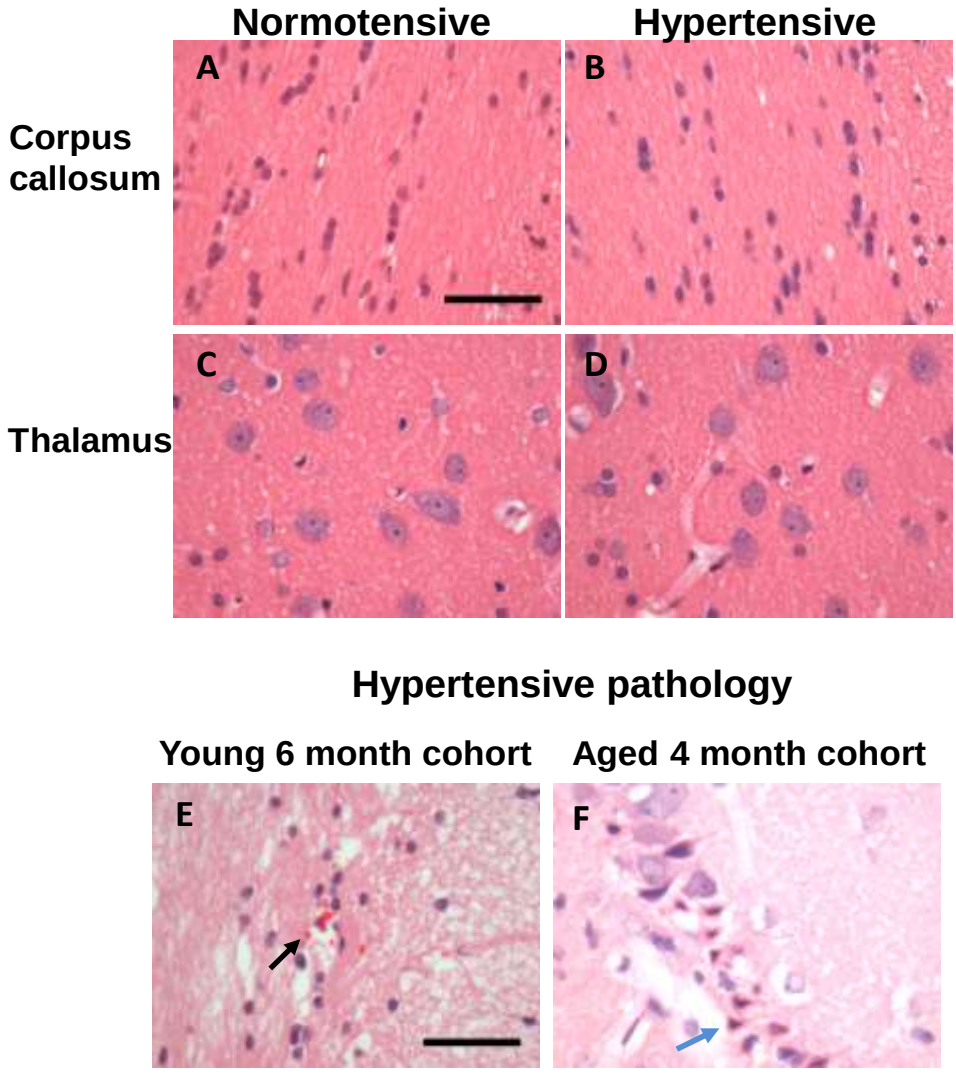

Figure 5.6: No overt structural alterations with hypertension

Representative images of the structural integrity of white and grey matter visualised using Haematoxylin and Eosin (H&E) histology. In several white matter tracts H&E was used to investigate the presence of tissue pallor, microbleeds and haemorrhages. (A) Normotensive and (B) hypertensive, images representative of no structural alterations within the white matter of all three cohorts investigated. Investigations in the subcortical thalamic region of (C) normotensive and (D) hypertensive animals found no overt alterations (images representative of no structural or neuronal damage) in all three cohorts investigated. Thus, hypertension did not induced overt structural alterations in the majority of animals investigated apart from one animal from the young 6-month cohort (E); which was observed to have small areas of microbleeds in the corpus callosum with pallor to the surrounding tissue. In addition one hypertensive animal from the aged 4-month cohort (F) was observed to have subtle evidence of ischemic damage to the neuronal perikarya (pink stained surrounding cells) shown by the blue arrow and surrounding areas of cellular vaculation within the hippocampus. Scale bar = 50µm.

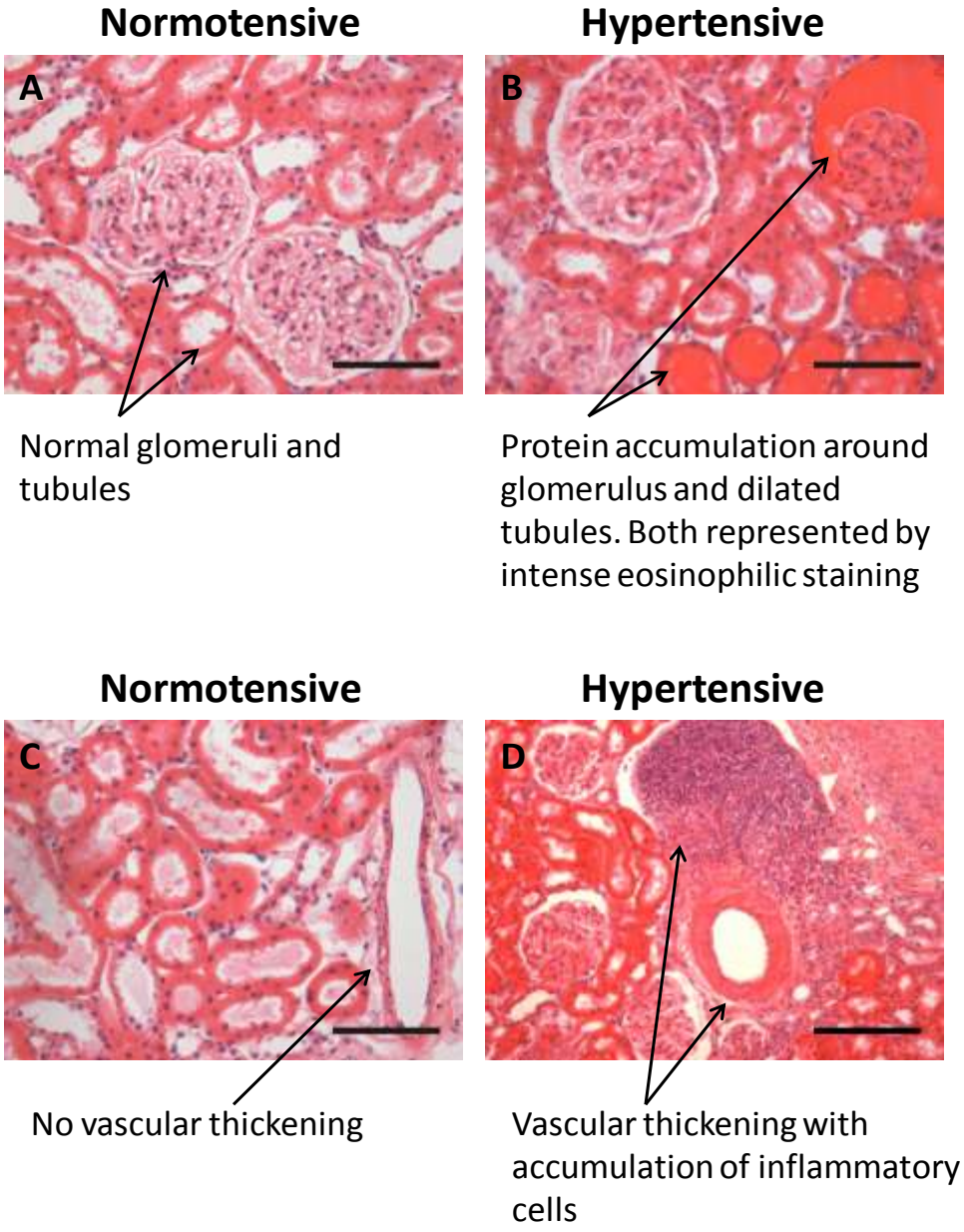

Figure 5.7: Kidney pathology

Representative images of structural alterations induced by I3C hypertension in the kidney, visualised by H&E, in agreement with previous findings reported in this model (Graciano, Mouton et al. 2007). (A-B) representative images of glomeruli structure; (B) hypertensive animals were found to have protein accumulated within the glomerulus and dilated tubules, (A) normal glomeruli in normotensive animals with no evidence of protein accumulation or tubule dilation. (C-D) representative images of vascular integrity; (D) hypertensive animals were found to have marked vascular thickening surrounded by proliferation of possible inflammatory cells when compared to normotensive kidneys (C). Scale bar= 100µm

5.3.5. Hypertension induced increased microglial activation

Iba1 was used to identify activated microglia within several regions of the brain. In chapter 3, Iba1 staining was used in a qualitatitive manner to investigate the association of microglia with the blood vessels. It was commented that along with hypertensive animals from the young 6-month cohort and the aged 4-month cohort there is evidence of increased numbers of vessels within specific regions as a whole, which was due to the following data. Immunopositive cells with a distinct cell body and surrounding processes were counted; processes without a cell body were not counted. In the young 4-month cohort there was no difference in the morphology of microglia between hypertensive and normotensive animals in all regions analysed. There was no significant difference in the number of microglia in white or grey matter regions examined between normotensive and hypertensive animals: corpus callosum ($|t|= 0.100$, df= 27, $p=0.920$; Figure 5.8E, Table 5.5), fimbria ($|t|= 1.83$, df= 27, $p=0.08$; Figure 5.8F, Table 5.5), internal capsule ($|t|= 1.91$, df= 27, $p=0.07$; Figure 5.8G, Table 5.5) and subcortical thalamic region ($|t|= 1.16$, df= 27, $p=0.26$ Figure 5.8H, Table 5.5).

In the young 6-month cohort there was no difference in the number of activated microglia within white matter tracts; corpus callosum ($|t|= 0.27$, df= 21, $p=0.82$; Figure 5.9E Table 5.6) and fimbria ($|t|= 0.12$, df= 21, $p=0.90$; Figure 5.9F, Table 5.6) in hypertensive animals when compared to normotensive. However, there was a significant increase in the number of microglia in hypertensive animals compared to normotensive in the internal capsule ($|t|= 4.02$, df= 21, $p<0.01$; Figure 5.9G, Table 5.6) and subcortical thalamic region where loss of MBP was observed

(|t|= 2.88, df= 21, p<0.01; Figure 5.9H, Table 5.6). These results show that with increased duration of hypertension there is a subcortical inflammatory response.

In the aged 4-month cohort there was a significant increase in the number of activated microglia between hypertensive compared to normotensive animals in the corpus callosum (|t|= 3.44, df= 18, p<0.01; Figure 5.10E, Table 5.7), internal capsule (|t|= 2.73, df= 18, p=0.01; Figure 5.10, Table 5.7) and subcortical thalamic region (|t|= 2.70, df= 18, p=0.01; Figure 5.10H, Table 5.7).The only exception was in the fimbria where no significant difference between normotensive and hypertensive animals was found (|t|= 2.25, df= 18, p=0.04; Figure 5.10F, Table 5.7). Hypertension with age provides evidence of globally increased microglia in multiple regions of the brain.

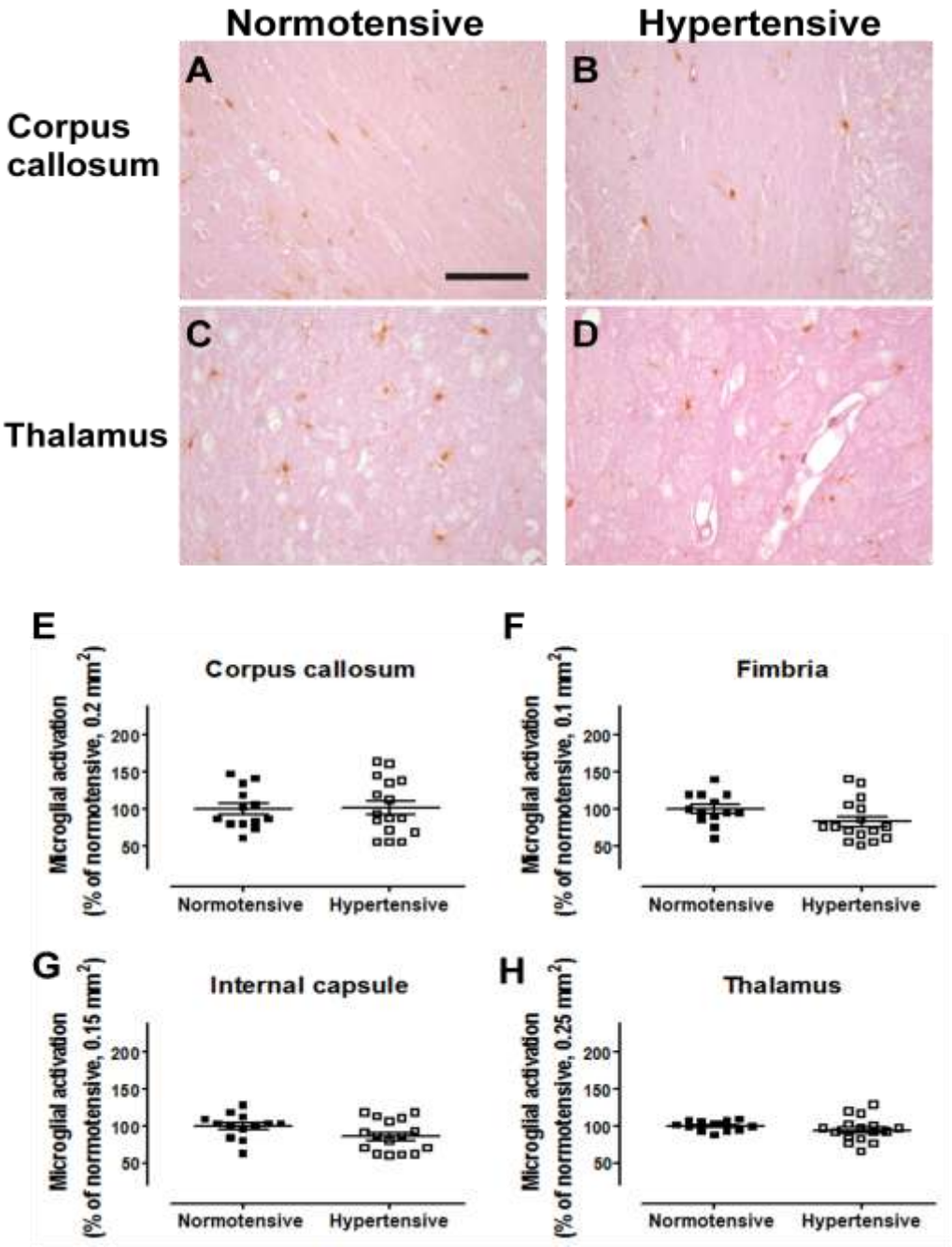

Figure 5.8: No change in the number of microglia in the young 4-month cohort

Representative images of microglia expression in the white and grey matter. Microglia visualised by antibody specific for IBA1 (brown) and counterstained with periodic acid schiffs (pink) vascular marker. In the corpus callosum there was no significant difference in the number of microglia between normotensive (A, E) and hypertensive animals (B, E). As in other white matter tracts investigated; (F) fimbria and (G) internal capsule. In the subcortical thalamic region the number of microglia were not significantly different between normotensive (C,H) and hypertensive animals (D, H). Scale bar = 50µm. Graphs show mean ± SEM.

IBA1 data for the Young 4-month cohort							
Number of microglia per region							
Corpus Callosum		Fimbria		Internal Capsule		Thalamus	
Normotensive	Hypertensive	Normotensive	Hypertensive	Normotensive	Hypertensive	Normotensive	Hypertensive
26.67	35.00	36.67	28.33	36.25	27.92	31.94	31.81
38.33	42.50	40.00	46.67	33.75	38.75	34.19	30.31
35.00	37.50	46.67	25.00	38.33	39.58	33.38	31.25
15.83	21.67	30.00	20.00	28.33	21.67	33.50	28.44
27.50	41.67	28.33	45.00	41.67	41.25	29.44	40.06
36.67	22.50	20.00	25.00	29.58	37.08	27.56	30.13
20.83	30.83	40.00	23.33	45.00	21.67	33.13	25.56
19.17	35.83	25.00	23.33	22.08	32.50	31.13	36.38
22.50	14.17	33.33	25.00	35.42	24.58	32.25	28.75
30.83	18.33	31.67	35.00	39.58	31.67	31.00	28.38
22.50	14.17	40.00	16.67	36.25	24.58	31.75	23.88
20.83	17.50	31.67	33.33	35.42	30.42	29.00	30.19
26.67	14.17	36.67	18.33	36.25	21.25	31.94	20.31
	22.50		21.67		20.83		23.81
	29.17		38.33		41.25		37.38
	24.17		18.33		30.42		26.06

Table 5.5: The number of microglia within the gray and white matter of the young 4-month cohort

The above table represents the raw numbers of microglia identified by IBA1 immunohistochemistry within several regions of the brain.

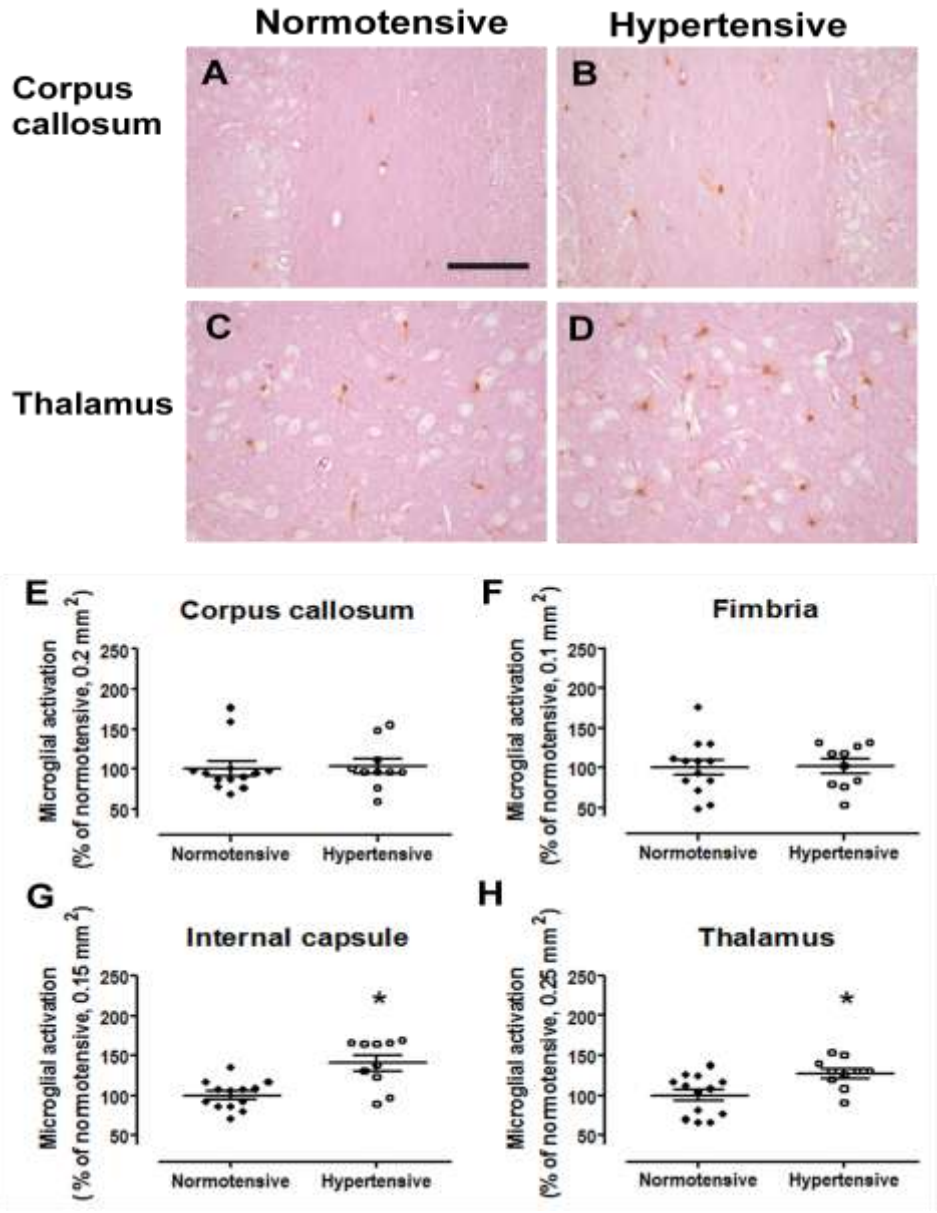

Figure 5.9: Increased number of subcortical microglia in the young 6-month cohort

Representative images of microglia expression in the white and grey matter. Microglia visualised by antibody specific for IBA1 (brown) and counterstained with periodic acid schiffs (pink) vascular marker. In the corpus callosum there was no significant difference in the number of microglia between normotensive (A, E) and hypertensive animals (B, E). The same finding was observed in the fimbria (F). An increased expression in the number of microglia was found with hypertension in the internal capsule (G). Similar finding in the subcortical thalamic region with increased number of microglia in hypertensive (D, H) when compared to normotensive animals (C,H). Scale bar = 50µm. Graphs show mean ± SEM. Significance * = $p \leq 0.02$ vs. Normotensive.

IBA1 data for the Young 6-month cohort							
Number of microglia per region							
Corpus Callosum		Fimbria		Internal Capsule		Thalamus	
Normotensive	Hypertensive	Normotensive	Normotensive	Normotensive	Hypertensive	Normotensive	Hypertensive
21.50	41.00	11.50	28.50	25.00	46.50	29.00	48.00
24.00	26.50	28.00	25.50	41.50	59.00	55.00	67.00
24.00	26.50	28.00	25.50	41.50	59.00	56.00	62.00
21.00	28.00	23.50	22.00	38.50	49.00	50.00	58.00
27.00	31.00	23.50	27.50	30.50	58.50	61.00	68.00
27.00	26.50	23.50	18.00	30.50	43.50	36.00	58.00
26.00	26.50	18.00	16.50	37.50	58.50	46.00	56.00
19.00	43.00	10.50	28.50	28.50	60.00	52.00	58.00
25.00	21.00	18.00	11.50	32.50	31.50	31.00	53.00
44.00	16.50	15.50	17.00	32.50	34.50	48.00	40.00
49.00		38.00		48.00		52.00	
28.00		20.00		38.00		29.00	
26.00		24.00		38.00		34.00	

Table 5.6: The number of microglia within the gray and white matter of the young 6-month cohort

The above table represents the raw numbers of microglia identified by IBA1 immunohistochemistry within several regions of the brain.

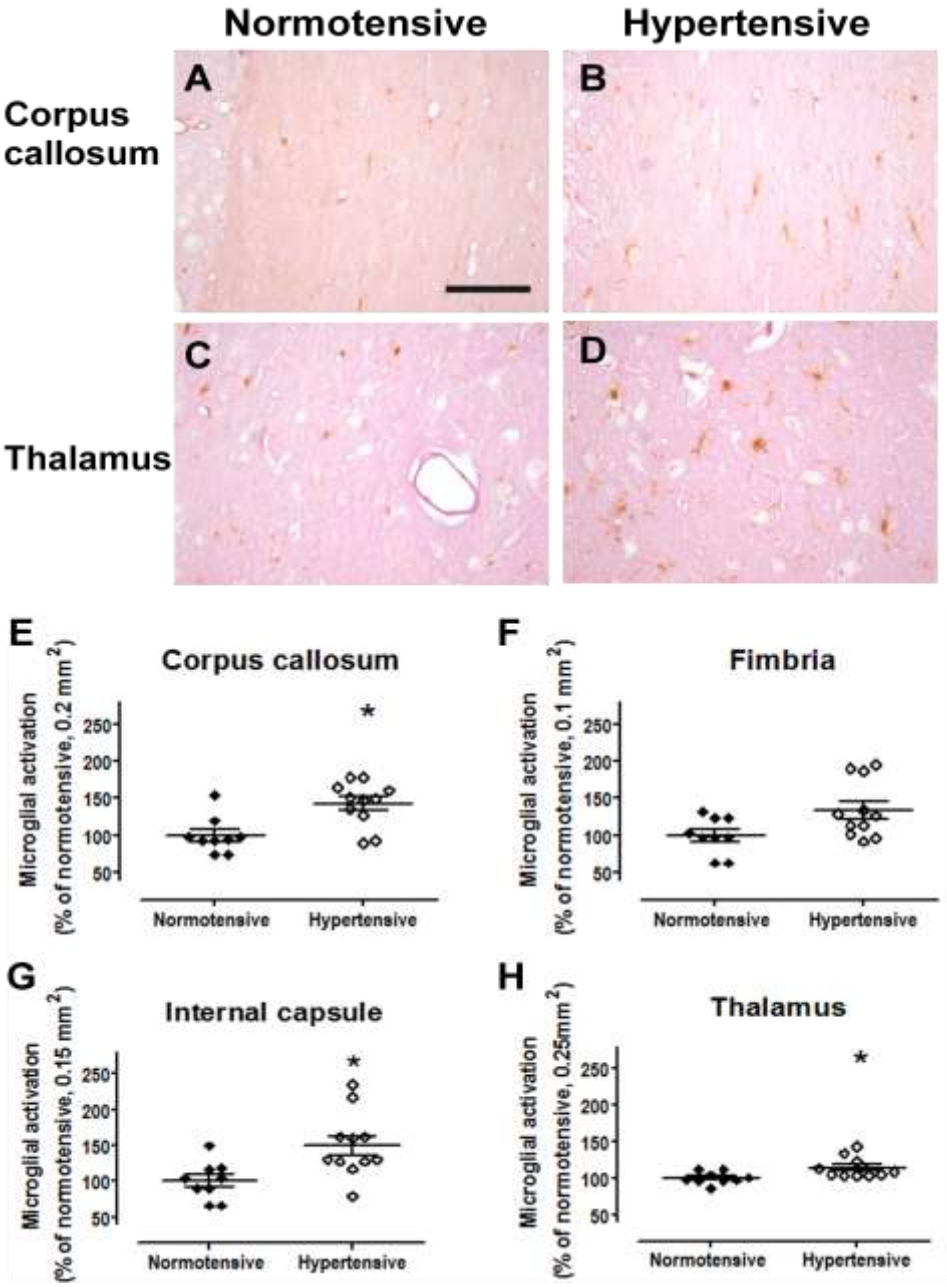

Figure 5.10: Increased numbers of microglia in the aged 4-month cohort

Representative images of increased microglia expression in the white and grey matter. Microglia visualised by antibody specific for IBA1 (brown) and counterstained with periodic acid schiffs (pink) vascular marker. In the corpus callosum there was a significant increase in the number of microglia between normotensive (A, E) and hypertensive animals (B, E), which was also found in the internal capsule but not in the fimbria (F). The number of microglia was also increased in the subcortical thalamic region in hypertensive animals (D, H) when compared to normotensive animals (C,H). Scale bar = 50µm. Graphs show mean ± SEM. Significance * = $p \leq 0.02$ vs. Normotensive.

IBA1 data for the Aged 4-month cohort							
Number of microglia per region							
Corpus Callosum		Fimbria		Internal Capsule		Thalamus	
Normotensive	Hypertensive	Normotensive	Hypertensive	Normotensive	Hypertensive	Normotensive	Hypertensive
27.50	42.50	18.50	24.50	42.00	75.00	33.25	46.50
21.00	25.00	12.00	35.50	48.50	60.00	39.00	36.00
21.00	42.00	12.00	17.50	48.50	55.00	33.75	42.75
27.50	26.00	18.50	18.00	42.00	60.00	39.00	40.00
26.50	45.00	18.50	25.50	54.50	76.00	36.25	36.50
26.00	41.50	23.50	18.50	31.00	102.00	34.00	36.00
26.00	38.00	23.50	37.00	31.00	76.00	33.50	49.75
33.50	46.00	25.00	36.00	55.50	109.00	30.00	39.25
43.50	50.00	19.50	21.50	70.00	61.00	35.00	37.75
	35.50		24.00		37.00		36.50
	50.00		21.50		61.00		36.00

Table 5.6: The number of microglia within the gray and white matter of the aged 4-month cohort

The above table represents the raw numbers of microglia identified by IBA1 immunohistochemistry within several regions of the brain.

5. Discussion

The present study suggests that sustained hypertension results in modest alterations to the brains WM. In particular evidence was provided to indicate subcortical myelinated axons may be vulnerable to the effects of hypertension. In addition a more pronounce widespread microglial response was found subcortically and in a number of white matter regions suggesting that there are underlying pathological changes occurring in response to hypertension.

The WM of the brain has been shown to be particularly vulnerable to cerebrovascular alterations, due to receiving its blood supply end of the line, from the penetrating arterioles. Thus the central focus of this study was to examine myelin integrity in response to hypertension in the young and aged brain. Myelin integrity in the present study was examined by measuring the intensity of an abundant myelin protein, myelin basic protein. Myelin alterations in this model were restricted to the subcortical thalamic region, with the majority of regions analysed found to be unaffected. These findings suggest a vulnerability of the subcortex to hypertensive pathology. In hypertensive patients, areas of the brain in which WML frequently occur such as the periventricular and subcortical regions of the brain, are those which have a marginal blood supply under physiological conditions (De Reuck, 1971; Marinkovic, 1986; Pantoni and Garcia, 1997a; Van Den Bergh, 1969). The subcortical thalamic region myelin may be vulnerable to hypertensive driven alterations due to a lack of vascular reserve, receiving its blood supply end of the line from the thalamopenetrating arterioles. These arterioles originate from the vertobasilar system and form few anatosomes, which would leave this region

sensitive to alterations in blood flow (De Reuck, 1971; Marinkovic, 1986; Pantoni and Garcia, 1997b; Van Den Bergh, 1969). The vulnerability of the subcortex has been identified in hypertensive patients demonstrating decreased thalamic blood flow during cognitive testing, which correlated with impaired performance (Fujishima et al., 1995b; Jennings et al., 2005). In addition, a previous chapter within this thesis found that hypertension alters cerebrovascular morphology. The susceptibility of subcortical WM was identified using MBP, although other components of the myelin sheath exist. MBP accounts for 30% of the total myelin sheath (Boggs, 2006) and has been shown to be essential for the formation of the myelin sheath with mutant MBP models lacking compact myelin (Carre et al., 2002; Readhead et al., 1990).

The subcortical myelin alterations found in this study were specific to the young brain. Surprisingly hypertension in the young 4-month cohort was found to be increased in the intensity of MBP expression, in comparison to normotensive animals, suggestive of hypermyelination. This result was consistent with the MRI data (Appendix E.1) showing myelin alterations consistent with increased myelin and this was also only found to be within the thalamus. The function of increased intensity of MBP is unknown in this study, but may represent an adaptive response of the brain to hypertension. Hypermyelination has been associated with hypoxic-ischemia in the prenatal condition of Status marmoratus, characterised by neuronal loss and myelin growth in the subcortex and basal ganglia (Yikilmaz and Taylor, 2008). However, increased myelin is not always a function of repair as was shown in hypermyelinating squirrel monkeys, where increased myelin was associated with cognitive decline (Lyons et al., 2004).

In addition previous studies within chapter 4 of this thesis identified altered gene expression of IGFBP within the subcortex, which regulates the expression of IGF. Overexpression of IGF has been found to cause hypermyelination in transgenic mice (Mozell and McMorris, 1991; Ye et al., 1995). The link between these findings and those of the present study may indicate a mechanism by which a relatively short duration of hypertension led to alterations in vascular signalling, which may have caused increased myelin.

Although the mechanisms of increased myelin at this time point are not understood, the findings are validated by imaging, which was undertaken in-vivo prior to study termination. MRI imaging provided evidence of increased magnetisation transfer ratio (MTR) within the same subcortical region measured in immunohistochemical assessment (Appendix E.1). MTR measurements are deemed more sensitive than the traditional T2 weighted MRI, and represents the ability of molecules located within the tissue matrix of the brain to exchange magnetisation with the surrounding water molecules. Alterations in the MTR can reflect changes in the structure of myelin or axons (McDonald et al., 1992). Since no evidence of axonal disruption was found, using other MRI matrices and no change in the microstructural organisation analysed by diffusion tensor imaging, it is likely that the increased MTR is reflective of increased myelin. However, to fully discard the presence of axonal alterations further investigation into axonal pathology such as electron microscopy or immunohistochemistry would have been required.

However in the present study, with increased duration of hypertension, there was a reduction in MBP intensity in the subcortical thalamic region, suggesting a loss of myelin. In both human and animal models, mechanisms underlying WML are

still unknown but have been linked to vascular remodelling and reductions in blood flow (Fisher, 1969; Lin et al., 2000; Lin et al., 2001; Wakita et al., 1994, 1995). Myelination is an energy-rich mechanism and not only has hypertension been shown to impair subcortical thalamic activity dependent blood flow, but there is also evidence for decreased glucose utilisation (Fujishima et al., 1995b). Within this study the initial increase in myelination may deplete energy stores required for the maintenance of the myelin sheath, coupled with the possible underlying vascular alterations, which have been associated with hypertension in humans and experimental models (Alistair, 2002; Lin et al., 2001; Pantoni and Garcia, 1995). In turn, a previous study within this thesis examining differential gene expression within the subcortex at the same duration of hypertension found alterations in energy metabolism. In addition, hypertension was also found to alter the structure of the cerebrovasculature, with vessels appearing smaller and string-like. This may have left the brain with insufficient blood flow and energy to maintain structural integrity. In turn, the hypermyelination found with the shorter duration of hypertension may have destabilised the myelin sheath predisposing this region to myelin loss.

Additionally, as shown in the previous chapter gene expression was analysed in a separation cohort ran for the same duration. Although there was no alteration found in MBP gene expression there was differential expression of the IGFBP gene, which was found to be differentially expressed with hypertension. With the same duration of hypertension in the same region, IGFBP was found to be upregulated, which would have lead to a downregulation of IGF. Literature has shown that decreased IGF expression leads to decreased myelination and loss of myelin integrity (Ye et al., 1995). The present study may have identified a novel signalling pathway,

which may be altered as a result of hypertension. The lack of alterations in MBP gene expression may be due to the microarray study sampling a larger area including multiple regions - inclusive of the entire subcortex, including parts of the internal capsule etc. However, a limitation of this study is that MBP levels were not further investigated by other means of analyses such as western blotting. As the significance difference found was strong and the majority of alterations found within this cohort in chapter 3 were vascular, it was decided that the tissue suitable for western blotting was processed into a vascular enriched homogenate to yield more of an indication as to the vascular alterations observed.

Overall, in the young adult brain hypertension caused alterations in subcortical MBP expression, which within the young 4-month cohort corresponded to MRI findings. At the outset it was hypothesised that in the aged cohort, hypertension would cause loss of the integrity of the brains WM. Thus, in this study it was surprising to find no evidence of alterations to myelin with hypertension in the aged 4-month cohort. In human studies there is an increased prevalence of WML with age (Van Swieten et al., 1991). However, the age of animals examined within this study is more akin to midlife hypertension in humans, which has been associated as a risk factor and predictor for WML in later life (Launer Lj, 1995; Swan et al., 1998; Vuorinen et al., 2011). It is interesting that with age there was no evidence of increased intensity in MBP, as found in the hypertensive animals from the young 4-month cohort. Since myelination is associated with higher glycolytic levels, the lack of hypermyelination in the aged brain, may represent an underlying decrease in energy availability, which is exacerbated by hypertension (Fern et al., 1998; Morland et al., 2007). In turn, studies have shown a decrease in IGF levels with age which

may also prevent increased myelination (Florini et al., 1981; Florini et al., 1985). In addition, there may have been methodological issues within this study, as one would expect alterations to occur with normal ageing. These alterations may have caused an increased variability in the study, thus the power of animals used may have not been sufficient to uncover subtle alterations in myelin integrity caused by hypertension. Prior to the start of this study power calculations were calculated based on previous findings within this thesis found in the young brain, but due to the novelty of this study examining animals at this age and lack of literature examining a similar insult to based calculations on, the power calculations may have underrepresented the number required.

However, there are minimal alterations found to myelin integrity in each cohort examined. In support of this, a recent study presented findings in COX10 deficient mice, which fail to metabolise glucose. Remarkably these mice have demonstrated intact CNS myelin, with evidence that oligodendrocytes have the ability to yield sufficient energy from a concurrent increase in lactate (Funfschilling et al., 2012). Therefore, under normal conditions as one would expect the brain has adaptive mechanisms in place to protect the myelin sheath. However, with prolonged duration of hypertension in the young 6-month cohort, which was previously shown to exhibit morphological alterations to the vascular structure, the adaptive response is insufficient to prevent alterations in myelin integrity. This protective response of increased lactate was also found to be dampened in the CNS grey matter myelin of the COX10 mutant mice, which may also explain the susceptibility of the subcortical myelin within the present study. Overall, this study found that hypertension leads to minimal alterations to the brains myelin. These observations are not in agreement

with previous findings in the SHR and SHRSP models - in particular none of the studies had observed hypermyelination. In general, previous studies in the SHR model have provided evidence of subtle WML, occurring firstly in the hippocampus and striatum at around 20 weeks of age (Lin et al., 2001; Sabbatini et al., 2001), which progressed to small focal areas of cystic and rarefied WM in the cortex and subcortex (Hazama et al., 1977). Imaging studies have shown a subtle increase in T2 weighted MRI measurements in the corpus callosum and internal capsule which represent small areas of decreased MBP intensity (Yang et al., 2011). The presence of increased myelin has not been found within the brain of the SHR model, although it has been reported in the aortic depressor nerve of mature SHRs (Fazan et al., 2005). Even though evidence has found that the SHRs have WML these are still relatively subtle, but as to be suspected, due to the increased severity they are more prevalent, than those found in the present study.

On the other hand, the SHRSP model has provided stronger evidence of WML throughout many regions of the brain; corpus callosum, anterior commissure, internal capsule and caudate at 20 weeks (Lin et al., 2001). These lesions have been shown to increase in severity with age, with WM fibres becoming dispersed and rarefied. The SHRSPs also show increased presence of haemorrhagic and cystic lesions in both the cortex and subcortex (Fredriksson et al., 1985; Fredriksson et al., 1988; Ogata et al., 1982), and by 1 year of age there is an increased frequency of bilateral WML (Henning et al., 2010). However, ischemic lesions have been found in 80% of SHRSPs by 30 weeks, and studies have also found ischemic lesions as early as 12 weeks (Tagami et al., 1987; Yamori and Horie, 1977). In turn, at 20 weeks when WML are observed, the SHRSP model exhibits malignant systolic blood

pressure of >220 mmHg. Therefore, WML occurring in the SHRSP models cannot be exclusively attributed to hypertension with a predisposition for stroke and malignant hypertension more likely. To further justify this, a recent study carried out to investigate aged stroke free SHRSPs found no evidence of WML (Brittain et al., 2012). Thus, the levels of hypertension and predisposition for the development of stroke likely explain differences in findings between the SHRSPs and our model.

The present study demonstrates that the myelin component of white matter is not overtly affected by hypertension in isolation, when modelled in a sustained manner and compared to genetically matched controls. This study highlights region specificity of hypertension and initial mechanisms of myelin alterations, which could be a target for therapeutic intervention. However, a limitation of the present study is that it only examined one component of the myelin sheath, myelin basic protein. Myelin associated glycoprotein (MAG), an architectural protein connecting the axon and myelin sheath has been shown to be lost prior to alterations in myelin or axonal integrity in response to hypoperfusion (Reimer et al., 2011). It is unknown within this study what effect hypertension has on the other components, which may impair the function of the brains WM.

Axons are also a critical component of WM for transmission of signals from neurons. Previously it was presumed from MRI findings that axonal integrity was unaltered with hypertension. Axonal disruption was assessed in the present study using APP immunohistochemistry, since under normal conditions APP is trafficked along the axon but when there is axonal disruption accumulations of APP are formed, which appear as axonal bulbs (McKenzie et al., 1996). Overall the present study found no evidence of axonal pathology in the majority of animals from each of

the cohorts examined. In a minority of hypertension animals from the young 6-month cohort, there were small subcortical areas of axonal pathology, which occurred after the onset of myelin alterations. Overall there was no significant difference in axonal integrity when compared to normotensive animals. Small areas of subcortical axonal pathology were also found in a subset of animals from the aged 4-month cohort. Interestingly these findings of axonal pathology in both normotensive and hypertensive animals represent alterations which occur naturally with age and may make it more difficult to unmask those alterations caused exclusively by hypertension. In addition lack of axonal damage may be a result of the myelin sheath, as it protects the axon from exposure to harmful stimuli, therefore, the subtle axonal pathology found in hypertensive animals may represent exposed axons that have lost their myelin sheath. It is not surprising that the present study did not find significant evidence of axonal pathology with hypertension as studies in humans examining WML have found axonal pathology only occurs in small and variable areas of the brain after severe demyelination (Van Swieten et al., 1991). Data from the present study is in agreement with a prior hypertensive imaging study carried out in 4 month old SHR, which failed to provide evidence of axonal pathology (Yang et al., 2011). Furthermore, previous literature in the SHRSP model provided evidence that within ischemic lesions axonal integrity is unaffected (Fredriksson et al., 1988).

The myelin of the WM is formed by oligodendrocytes. These cells project processes, which enwrap the intermodal segments of axons to form the myelin sheath allowing effective propagation of axon potentials, whilst sheltering the axon from harmful stimuli (Bunge et al., 1961; Mayhan et al., 1987; Nave, 2010). This energy-rich process of myelination and its maintenance is carried out in an energy limited

environment, making oligodendrocytes vulnerable to periods of energy disruption (Todorich et al., 2009). Thus the numbers of oligodendrocytes were measured within the subcortical thalamic region of each cohort to determine whether they were altered in response to hypertension. We found no evidence of alterations within the number of oligodendrocytes in each of the cohorts investigated, but did not use markers to investigate the integrity of corresponding myelination processes or take into account other populations of oligodendrocytes such as precursors of immature oligodendrocytes. However, our data is in agreement with findings in the SHR model observing minimal alterations to the WM, which were not accompanied with alterations in the number of oligodendrocytes (Yang et al., 2011). It does need to be addressed that there appears to be an overall increase in the number of oligodendrocytes in the aged cohort. This was not statistically analysed as the three cohorts were set up under differing conditions and both immunohistochemistry and analysis was carried out at the different times, thus it is unknown whether there is a difference with age or due to experimental conditions. The data in these studies suggests that hypertension in isolation causes minimal myelin alterations and these alterations do not affect the number of oligodendrocytes or the integrity of axons in the brain.

The present study did not address the effect of hypertension on the astrocytic component of the WM. Proliferation of astrocytes has been observed in the SHR model at >6 months of age in the cortical and subcortical WM (Tomassoni et al., 2004) after the development of WM pathology. Astrocytes have also been shown to be proliferated after >2 months of age in the SHRSP models in response to malignant hypertension (Hazama et al., 1995).

Overall the alterations caused by hypertension in this chapter and present chapters have been found to be modest. A non-specific qualitative investigation of the overall structural integrity of the brain was carried out using H&E staining to investigate if there were any signs of overt pathological structural alterations. H&E staining was used for the identification of ischemic damage to the neuronal perikaryia, microbleeds, haemorrhages and infarcts. Overall, hypertension did not lead to any overt structural alterations to the brain in each of the cohorts examined, aside from two hypertensive animals: one from the young 6-month cohort; observed to have small areas of microbleeds in the corpus callosum with overall tissue pallor and one animal from the aged 4-month cohort; observed to have signs of neuronal damage in the corpus callosom. It does need to be discussed that H&E is a very crude maker of neuronal damage and many other makers of ischemic neurons could have been used in this study. However, the H&E findings indicated that overall there was no evidence of ischemic neurons and overt structural pathology in hypertensive animals from each cohort when compared to their respective normotensive controls thus these findings were not further investigated. These findings are in disagreement with histological studies in the SHR and SHRSP models observing structural alterations to the brains gray and WM (Hazama et al., 1977; Knox et al., 1980; Ogata et al., 1980; Sabbatini et al., 2000). The SHRSP model has also been found to have areas of microinfarcts and bleeds, which frequently occur around the same time when stroke-like symptoms appear (Ogata et al., 1980). Overall the lack of overt alterations found with the previous study may be due to the induction of blood pressure in a slow gradual manner, unlike the SHRs and SHRSPs (Okamoto and Aoki, 1963; Okamoto, 1974). In turn, the vascular alterations found previously were relatively

subtle and one would not have expected these to have caused gross overt structural alterations, rather more subtle specific protein alterations within the most vulnerable regions of the brain such as the WM.

However, as shown in chapter 3 hypertension led to vascular inflammation, which was associated with structural alterations to the vasculature. In addition, differential gene expression also suggested that there was evidence that hypertension caused an inflammatory response. The present study chose to examine whether there is an increased inflammatory response in the WM of the brain, which might be related to the alterations to myelin. The presence of an inflammatory response was analysed by counting the number of microglia immunopositive for Iba1, which binds to the actin cytoskeleton of microglia and is expressed during proliferation, migration and phagocytosis (Ahmed et al., 2007).

Although hypertension induced minimal alterations to myelin and axons, there was a marked inflammatory response. There was no change in the number of microglia in the young 4-month cohort, but with increased duration of hypertension in the young 6-month cohort, there was a marked increased in the number of microglia in hypertensive animals when compared to normotensive. This increased inflammatory response was located within the subcortical thalamic region, which had loss of MBP and also the WM tract- the internal capsule.

In the aged 4-month cohort, an increased microglial response was found in multiple regions. Increased numbers of microglial cells were found to be induced by hypertension in the majority of regions analysed. In the aged 4-month cohort there was no evidence of alterations in myelin therefore microglia may be recruited in response to other pathological insults. These findings of increased microglia with

hypertension are in agreement with other experimental models of hypertension such as the SHR, SHRSP, renal models and Angiotensin II infused mice and may represent an early mechanistic feature of hypertension (Knox et al., 1980; Lin et al., 2001; Nag, 1984; Shi et al., 2010; Zubcevic et al., 2011).

Although it is unknown within this study whether the increased expression of microglial is a protective response or contributing to a pathological process it is notable that microglial respond more prominently than other components of the WM such as myelin, oligodendrocytes and axons, being described as sensors of pathology. In the transverse aortic coarctation (TAC) model of transient hypertension with marked alterations to CBF, microglia were found to be upregulated prior to structural alterations in the brain (Carnevale et al., 2012). Similar findings have been shown in other ischemia studies (Gehrmann et al., 1992) representing the early response of microglia to alterations in blood flow. Thus, the increased microglial response in the present study may indicate that microglia are acting as early sensors of damage.

Microglia can be recruited in response to structural and ionic alterations in the brain (Ransohoff and Perry, 2009). Previously within this thesis, structural alterations to BBB tight junction proteins were identified, which may have lead to alterations in ionic concentrations and caused an increased microglial response. These results were also accompanied by alterations in vascular structure found in both the young 6-month and aged 4-month cohort. Alterations in endothelial signalling and/or vascular structure may have altered blood flow. Mechanisms are in place to normalised blood flow, by neuron-astrocyte signalling. The glia often used Ca^{2+} signalling, therefore there may be an increased astrocytic Ca^{2+} signalling, in

attempt to normalised blood flow which may have also signalled to recruit microglia (Aloisi et al., 1997; DeWitt et al., 1998; Verderio and Matteoli, 2001).

In both human and aged animal studies there are alterations in transcellular transport across the BBB (Hunt et al., 2007; Mooradian, 1990; Mooradian et al., 1994; Mooradian et al., 1991; Mooradian and Smith, 1993), which although not examined may be exacerbated with hypertension in the aged cohort when compared to the young cohort therefore, leading to a marked inflammatory response. In turn, as previously shown with the axonal integrity data, aging alone predisposes the brain to structural alterations, which may lead to an exacerbated inflammatory response with hypertension. On the other hand, the increased number of microglial cells within the subcortical thalamic region is accompanied by loss of MBP in the young 6-month brain. As shown previously at this time point there are alterations to the structural integrity of the vasculature, with vessels appearing string-like and also evidence of BBB alterations. Overall, these alterations may predispose the brain to decreased blood flow, leading to loss of myelin and an inflammatory response.

However one limitation of the present study is within the induction of hypertension in the Cyp1a1 Ren2 rat model using Indole-3-carbinol, which have been the focus of several research studies as a possible cancer treatment due to it is anti-inflammatory properties (Bradlow et al., 1991; Higdon et al., 2007; Kojima et al., 1994; Weng et al., 2010). Therefore, the findings in this study may have been underplayed due to the method of hypertension induction. To find out the true extent I3C had on this study you would need to compare inflammatory levels between animals fed I3C and those not under normal conditions and in response to an inflammatory stimuli such as lipopolysaccharide (LPS), which has been used to

investigate the inflammatory response in other animal models (Espinosa-Oliva et al., 2013).

In conclusion, hypertension in the inducible rat model caused alterations in WM. These alterations were subtle in comparison to WM changes documented in human, which may be due to hypertension commonly coexisting with other vascular related risk factors such as diabetes, leading to an exacerbation of pathological findings and cognitive abilities (Messerli et al., 2007). To date work in this thesis has assessed the structure of the brain but has not examined the effect of hypertension on brain function.

6. The effect of hypertension on spatial reference and working memory

6.1. Introduction

For many decades hypertension has been associated with impaired cognitive function (Wilkie and Eisdorfer, 1971). However, as indicated previously there are many factors, which contribute to hypertension related alterations in the brain such as: duration, severity and onset of hypertension, added to many subjects being under the influence of treatment and the common coexistence of hypertension with other vascular risk factors, all of which can have a dramatic effect on study findings.

The gold standard models; SHR and SHRSP have provided a wealth of knowledge as to the possible actions of hypertension in the periphery and the brain, but have been less successful in providing robust evidence of an association of hypertension leading to cognitive decline, with one main limitation is the lack of genetically identical control rats.

The present study chose to examine cognitive function in the CYP1a1 Ren2 model in which sustained, controlled hypertension can be directly compared to age matched litter-mate normotensive controls. The forms of memory chosen to assess were spatial reference and working memory as these have been found to be impaired in humans with age (Salthouse, 2004; Salthouse, 2009).

6.1.2. Hypothesis

Hypertension will lead to impaired cognitive function in spatial reference and working memory.

6.1.3. Aims

The study described in this chapter aimed to investigate the cognitive abilities including spatial reference and working memory of hypertensive compared to normotensive Cyp1a1 Ren2 rats.

6.2. Methods

6.2.1. Subjects

Cognitive testing was carried out on young Cyp1a1 Ren2 transgenic rats; normotensive fed either a control diet (no 13C, n=12) or hypertensive (0.15% 13C, n=12) diet for a 4 month period as described in detail in chapter 2. Throughout the duration of the study blood pressure results followed the same profile as to those described in chapter 3 (Appendix G.1).

6.2.2. Assessment of spatial reference and working memory using the Morris water maze

The Morris water maze was carried out corresponding to protocols detailed in chapter 2.4. Prior to behavioural testing animals were habituated to handling due to weekly blood pressure measurements. In order to exclude animals with gross motor or visual impairments, a cued version of the water maze task was carried out, in which animals had to locate a cued visible platform. This cued version of the water maze also provided training of the task and was carried out for 3 trials per day for 4 consecutive days. No animals were excluded on this basis from the study. Spatial reference memory training began 3 days after the end of the cued task. Animals were allowed 4 trials per day for 5 days, to locate the hidden platform with an inter-trial interval of 10 minutes. Platform location was constant throughout the trials with animals starting location counter balanced to platform location. Probe tests were

carried out 10 minutes and 24 hours after the final trial of spatial reference memory task.

Further cognitive assessment was carried out using delayed matching to place task, 3 days after the end of the spatial reference memory task. As described in chapter 2.4.1.3, delayed matching to place task was carried out according to the protocol established by Steele and Morris, including alteration in platform location between trials (Steele and Morris, 1999). Animals were allowed 4 trials per day for 4 consecutive days, with trials 1 and 2 having an inter-trial interval of 20 minutes and trials 2, 3 and 4 having a shorter inter-trial interval of 15 seconds.

6.2.3. Hippocampal Myelin basic protein levels

As described in Chapter 5.3.1 MBP was used to assess the integrity of myelin. As performance of the above cognitive assessment is reliant on hippocampal function, MBP levels within the hippocampus were analysed by measuring the intensity of MBP in the young 4-month immunohistochemical cohort (same animals used in the chapter 5).

6.2.4. Statistical analysis

Behavioural data was analysed using two-way ANOVA with repeated measures. Hippocampal MBP data was analysed by student's t-test, significance $p<0.01$.

6.3. Results

6.3.1. Blood pressure

Hypertension was induced by the addition of I3C as described previously (Chapter 2.2). The blood pressure followed the same profile as the young 4-month cohort examined for immunohistochemistry described in chapter 3 (Appendix G.1).

6.3.2. Hypertension does not induce gross motor or visual impairments

Initially rats were trained in a cue task with a visible platform, showing that sensory function and basic learning were not impaired. There was no significant difference in performance between hypertensive and normotensive groups ($F_{(1, 21)}$ = 0.46 p=0.51; Figure 6.1). In both groups performance improved significantly across days ($F_{(4.325, 90.809)}$ = 3.79, p<0.01; Figure 6.1) and escape latencies averaged less than 10 seconds by the end of training. Swim speed measured across all trials did not differ between normotensive (28.11 ± 0.38 cm/s) and hypertensive (27.92 ± 0.36 cm/s) animals ($F_{(1, 21)}$ = 0.21 p = 0.65).

6.3.3. Spatial reference memory is conserved after hypertension

Spatial reference memory was investigated using the Morris water maze. Animals were given 4 trials per day to locate a hidden, non-cued platform, which remained in a constant location throughout the trial. There was no significant difference between normotensive and hypertensive rats, in the latency to locate the hidden platform across training ($F_{(1, 21)}$=1.08, p=0.31; Figure 6.2A). Both groups of animals demonstrated improvement in the ability to learn the location of the platform. The latency of both normotensive and hypertensive animals significantly decreased over the training period ($F_{(19, 399)}$=5.48, p<0.001; Figure 6.2A). In addition,

normotensive and hypertensive animals showed a similar preference for the quadrant in which they had been trained when memory was probed at 10 minutes (group x quadrant interaction, $F_{(3, 63)}$=0.75, p=0.53; Figure 6.2B) and 24 hours (group x quadrant interaction, $F_{(3, 63)}$=0.45, p=0.72; Figure 6.2C) after the final trial of spatial reference training.

6.3.4. Hypertension did not impair performance in the delayed matching to place task

In the delayed matching to place task, the location of the hidden platform changed each day. This was carried out to assess working memory capabilities in hypertensive animals when compared to normotensive controls. There was no significant difference in performance between normotensive and hypertensive animals ($F_{(1, 21)}$=0.83, p=0.37; Figure 6.3) but the animals did improve over time with latency to complete the task decreasing.

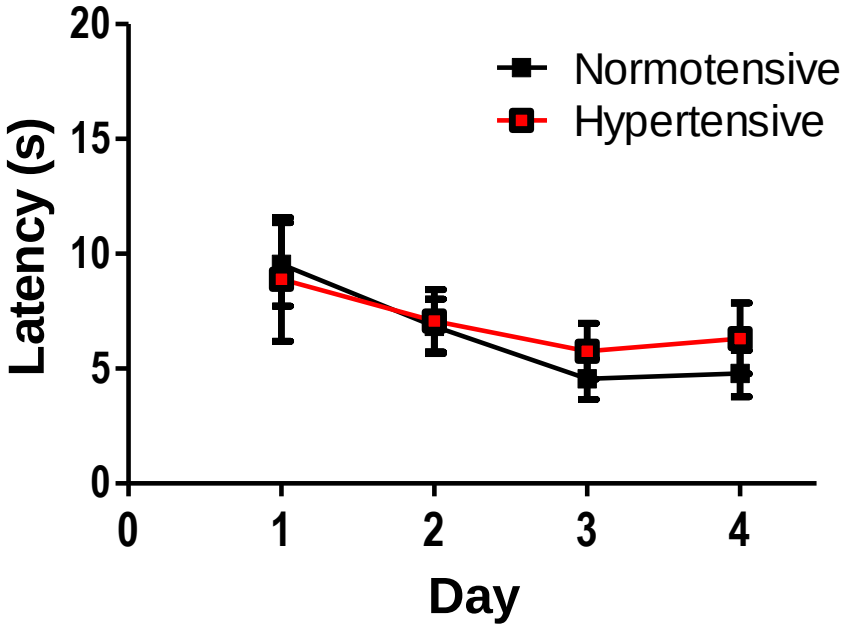

Figure 6.1: Cue task performance in the young 4-month cohort
There was no significant difference in performance between normotensive and hypertensive animals over the training period of the cued version of the water maze. Both groups improved their performance overtime. Graph shows mean ± SEM.

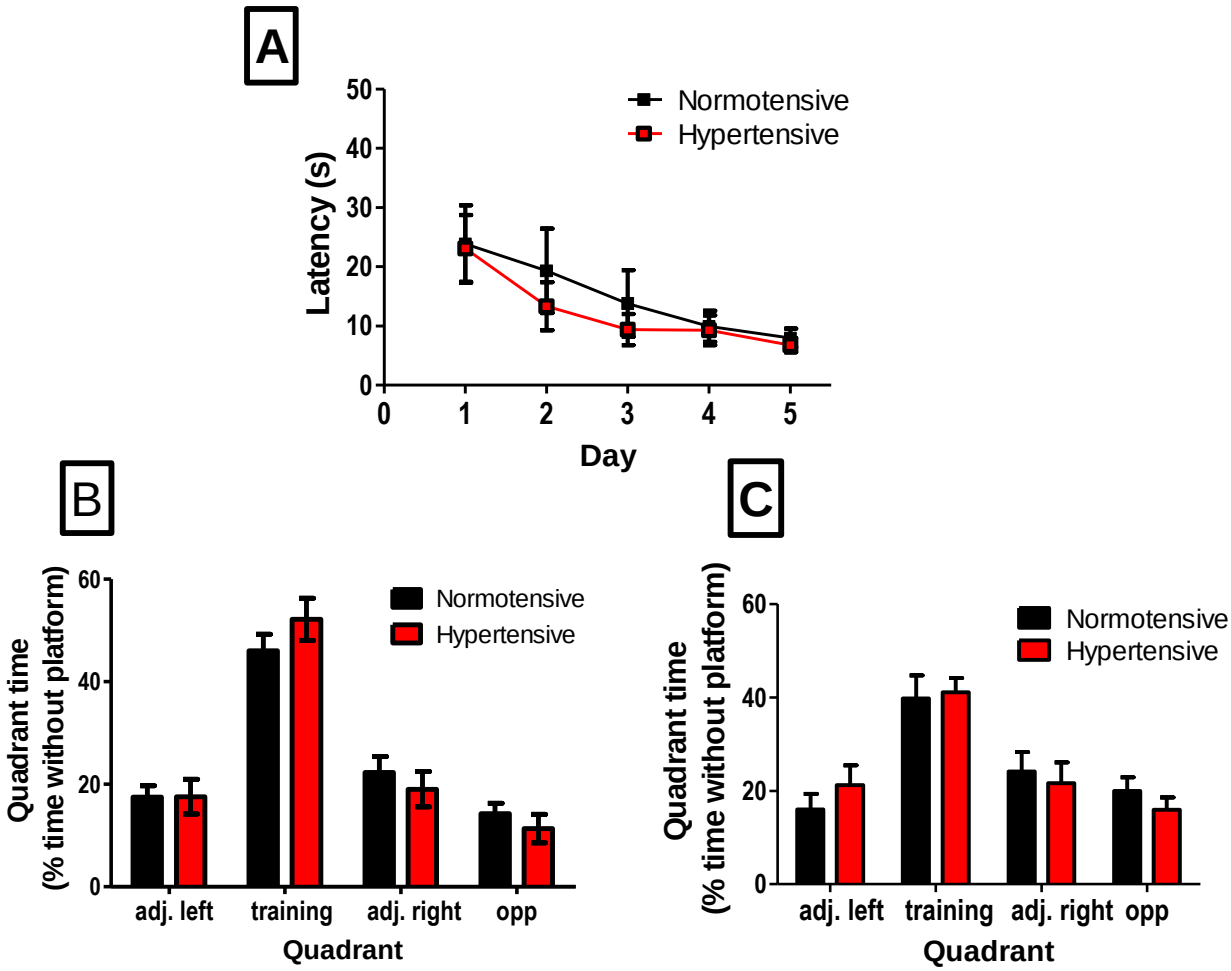

Figure 6.2: Water maze performance during spatial reference memory trials
There was no significant difference in the latency to locate the hidden platform between normotensive and hypertensive animals (A). Both groups displayed equal retention of platform location at 10 minutes (B) and 24 hours (C) after training. Graphs show mean ± SEM.

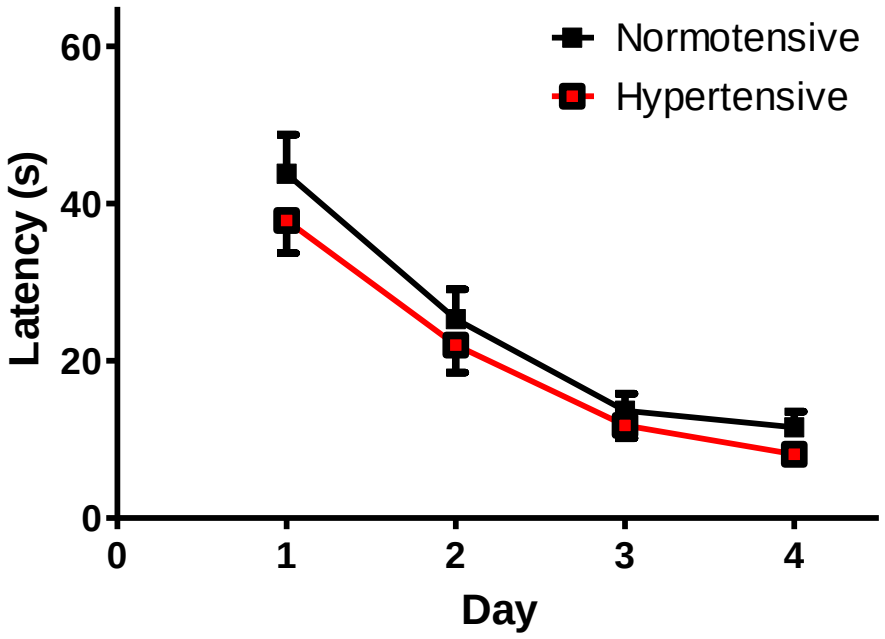

Figure 6.3: Water maze performance during delayed matching to place trials
There was no significant difference in performance during delayed matching to place task in hypertensive when compared to normotensive animals. Both groups show an equivalent decreased latency to complete the trial during the task. Graph show mean ± SEM.

6.3.4 *Hypertension did not cause alterations to hippocampal MBP levels*

The integrity of myelin was investigated by analysing the intensity of MBP staining. Although chapter 4 found evidence of hypertension leading to an increased intensity of subcortical thalamic MBP in the young 4-month cohort, the intensity of hippocampal MBP was not significantly different between normotensive and hypertensive animals (Figure 6.4, $|t| = 1.508$, $df = 26$, $p = 0.668$), representing at this time point hypertension does not lead to alterations to hippocampal MBP.

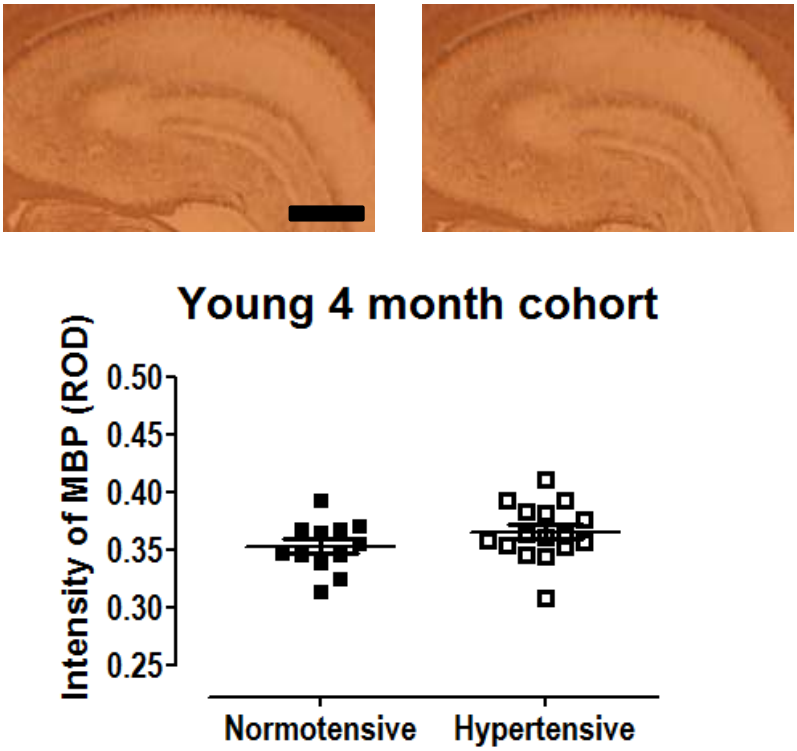

Figure 6.4: MBP levels in the Hippocampus

Representative images of MBP levels within the hippocampus of the young 4-month cohort. MBP was visualised using DAB immunohistochemistry using an antibody for anti-MBP. It was found that overall there was no significant different in the intensity of MBP (measured by ROD) between hypertensive and normotensive animals. (scale bar = 200µm).

6.4. Discussion

In humans, hypertension is a risk factor for the development of age related cognitive decline (Kilander et al., 1998b). A previous study within this thesis found that hypertension caused modest alterations to the WM with more marked alterations observed to the cerebrovasculature. The present study demonstrated that sustained hypertension did not lead to alterations to spatial reference and working memory at a time when modest subcortical WM alterations were evident

Firstly spatial reference memory was assessed using a standard Morris water maze protocol (Morris, 1981). All animals were trained and assessed for the presence of overt motor and visual deficits. Due to the albino strain of these animals there was a concern that there may be underlying visual deficits and additionally since no behavioural assessments had been previously carried out in this model, at this severity of blood pressure, it was unknown at the onset if the animals could complete the water maze task. However, during the cued task all animals were able to swim equally well and at comparable speeds and showed positive evidence of an ability to learn to locate the hidden platform. At this point there was no indication that the hypertensive and normotensive animals performed any differently during the training phase and all animals learned the task equally well.

After the animals had shown suitable performance in the cued task, spatial reference memory was assessed. It was found that there was no difference in the performance of hypertensive and normotensive animals in the spatial reference memory task. All animals were found to learn the task, with the latency of platform location decreasing over time and the following probe trial showing evidence of quadrant preference. These findings are suggestive that hypertension at this duration,

where initial structural alterations to myelin and additionally endothelial signalling are found, are not sufficient to alter cognitive function. Similar to the present finding, a study in 3 month old SHRs have found no evidence of impaired spatial reference memory, with SHRs found to perform the task better than the WKY (Widy-Tyszkiewicz et al., 1993).These findings are not exclusive to the Morris water maze, with investigations carried out using the radial arm maze in SHRs aged 3-4 months and 16-17 months showing no evidence of impaired spatial reference memory in the young SHR (Mori et al., 1995). However, other studies have shown evidence of impaired spatial reference memory in SHRs aged 4 weeks (Gattu et al., 1997b), 12 weeks (Gattu et al., 1997a) and 15 months (Terry et al., 2000). Interestingly these findings suggest that the SHRs have impaired cognitive function prior to the development of hypertension at 4 weeks, which may indicate evidence of background genetic differences between SHRs and WKYs (Gattu et al., 1997b). In turn, the examination of spatial reference memory in SHRSPs, SHRs and WKYs, found that spatial reference memory is impaired in SHRSPs but not SHRs or WKYs (Matsuo, 2007). Therefore, the evidence in SHRs and SHRSPs is overall difficult to interpret as there appear to be differences in strain performance. In fact, it has been reported that WKY exhibit altered and erratic swim speeds (Diana, 2002; Knardahl and Sagvolden, 1982; Randich and Maixner, 1981; Robertson et al., 2008; Wyss et al., 2000).

To further investigate cognitive function, the delayed matching to place paradigm was carried out to examine working memory. It was found as with spatial reference memory that hypertension did not cause alterations to the performance measured by latency to located the platform during the delayed matching to place

task. This task was chosen as it is more taxing on memory performance, as the location of the platform is moved each day, meaning that animals are required to relearn the location of the platform and more subtle difference can be uncovered. The protocol used for the present study was devised by Steele and Morris and allowed a longer inter-trial interval after trail 1, for encoding of the new platform location (Steele and Morris, 1999). There was no evidence of any difference in the performance between hypertensive and normotensive animals during the task as a whole or even between trials. In support of the present findings many studies have shown in the SHR model that there is no impairment in working memory but a superior performance when compared to normotensive WKY (Robertson et al., 2008; Wyss et al., 1992). However, there are also studies in the SHR model, which have found evidence of impaired working memory (Hernandez et al., 2003; Mori et al., 1995; Nakamura-Palacios et al., 1996; Wyss et al., 2000). Robertson et al., provided evidence that working memory is impaired in the SHR models when compared to the normotensive Sprague Dawley strain. However, WKY control animals also showed impairment in performance, which was comparative to that found in the SHR model, thus impairments may be not due to hypertension and as indicated previously can be strongly influence by control strain comparisons (Robertson et al., 2008).

The present study chose to examine cognitive function using the Morris water maze. As can be seen in table 6.1, key studies in the SHR and SHRSP have examined spatial memory using the Morris water maze and Radial arm maze with relatively similar results. The rational for choosing the Morris water maze in the present study was to avoid any alteration to the dietary induction of hypertension. Since the radial arm maze relies on food reduction and reward, as a stimulus, this may have led to

alterations to blood pressure levels, as the hypertension is induced through dietary admission.

Overall, the present study found no evidence of any alterations to spatial or working memory, even though there are alterations to the myelin integrity and endothelial signalling. The previous chapter presented alterations to the integrity of myelin within the subcortex; however the hippocampus is a crucial structure in water maze performance (Macdonald et al., 1973; Morris, 1981; Steele and Morris, 1999). Within this chapter it was also shown that hypertension does not cause an alteration to the intensity of hippocampal MBP, thus the lack of cognitive alterations observed within the functions assessed may be due to an overall lack of structural alterations. However, thalamic lesion studies have shown a key role for the integrity of thalamic nuclei in performance of spatial reference memory (van Groen et al., 2002a; van Groen et al., 2002b). In studies of cognitive function in humans with hypertension it has been shown that there are impairments in spatial memory, working memory, attention, psychomotor abilities and perceptual skills (Elias et al., 2004; Waldstein, 2003), many of which can be associated a disconnection to the cortical-subcortical circuits (Bonelli and Cummings, 2007; Charlton et al., 2006; Jellinger, 2007). However, the alterations found within the present study were relatively subtle and were not sufficient to cause any impairment in the cognitive functions assesed. Therefore, this may suggest that in the young brain the structural alterations cause by hypertension are not sufficient to cause a functional impairment.

Another limitation of the present study is that the effect of hypertension on cognitive function was studied in the young brain. The rationale for investigating cognitive function in the young brain was to decipher the role of hypertension at a

time point where initial structural alterations had appeared. The majority of human studies associate hypertension in midlife as a predictor of cognitive decline in late-life (Elias et al., 2004; Haan Mn, 1999; Kilander et al., 2000; Piguet et al., 2003; Reinprecht et al., 2003; Tzourio et al., 1999). Ageing has also been shown to lead to alterations in spatial learning and memory in animal models (Gallagher and Rapp, 1997). Arguably, the young brain may be able to adapt to hypertension induced alterations. Thus future studies in the current model should focus on examining cognitive function with hypertension in the aged brain.

Overall the present study provided evidence that hypertension does not lead to functional alterations in spatial and working memory. The major conflicting factor in human literature is the coexistence of hypertension with other vascular risk factors such as diabetes. Research into ageing has linked vascular risk factors such as diabetes and obesity to age-related cognitive decline (Kilander, 2000). It may be an additional effect of vascular risk factors, which lead to cognitive decline. In turn, the lack of cognitive impairment found in the present study may be reflective of the overall impact on hypertension on the structural integrity of the brain. This thesis found that hypertension leads to alterations to the cerebrovasculature and WM but these were relatively subtle and region specific. Thus it appears from the present study and other findings within this thesis that hypertension when studied in isolation, in a controlled sustained manner caused subtle alterations to the brain, which may be exacerbated with the addition of other vascular risk factors.

Reference	Cognitive assessment	Age	Number of animals	Evidence of cognitive impairment
Mori et al 1995	Radial arm maze	3-4 months and 16-17months	19 SHR 8WKY	- : in young +: in aged
Wyss et al 1992	Radial arm maze	3 months 12 months	6-8 per group Sprague Dawley (SD) was used as a normotensive control strain	-: in young +: in aged
Nakamura-Palacios et al 1996	Radial arm maze	**3 months**	**12 SHR** **11 WKY**	**+**
Hernandez et al 2003	Radial arm maze	**3 months** **9 months**	**9-12 per group**	**+**
Gattu et al 1997 part I and II	Morris water maze	**4 weeks** **12 weeks**	**9-12 per group**	**+/-: only impairment in reversal memory in the young animals** **+: impairment in both spatial and reversal memory in aged animals**
Terry et al 2000	Morris water maze	**15 months**	**12 per group**	**+**
Widy-Tyszkiewicz et al 1993	Morris water maze	**Exact age not reported but testing was carried out when animals weighed 260-320g**	**8 SHR** **19 Wistar**	**-**
Robertson et al 2008	Morris water maze	6-7 weeks	12-14 per group	+:SHR performed worse than SD strain -: No difference in performance between SHR an WKY

Table 6.1: Summary of key behavioural studies in the SHR model

Findings from the SHR model using either the Radial arm or the Morris water maze. + = cognitive impairment in the hypertensive model, - = no cognitive impairment in the hypertensive model. In general the results of cognitive assessments using the radial arm and water maze are inconsistent in the SHR model with some studies showing evidence of cognitive impairment are others not at similar ages.

7. General Discussion

7.1. Summary

The findings within this thesis provided insight as to the role of hypertension in isolation, induced in a controlled sustained manner. Hypertension was found to cause alterations to the cerebrovasculature consisting of: altered endothelial signalling, vascular remodelling and vascular inflammation. The cerebrovascular alterations were accompanied with differential gene expression of many growth factors, ion channels, energy metabolism and inflammatory related genes. Followed by evidence that hypertension caused modest alterations to the WM, in the young brain, which showed a specific predisposition to the subcortex. Accompanied with these alterations there was evidence of hypertension inducing a marked inflammatory response within subcortical grey and white matter tracts in the young brain and additionally several WM regions and subcortical grey matter in the aged brain. To provide a functional assessment of hypertension, cognition was assessed examining spatial reference and working memory, at the earliest point when structural alterations were observed but was found to be unaffected. This finding is suggestive that initial alterations caused by hypertension do not have a functional effect on the brain functions assessed. The alterations found within this thesis support several aspects of the hypothesis in that hypertension leads to structural alterations to the cerebrovasculature and WM, in the young and aged brain at varying severity but there was no evidence of cognitive impairment.

Interestingly this thesis identified several signalling pathways which appear to have been altered in part due to hypertension. As described in the introduction,

previous literature had associated hypertension with alterations to the MAP-Kinase pathway, in particular eNOS expression (Lassegue et al., 2001; Ungvari et al., 2003). In the present study eNOS is found to be altered with hypertension in the young and aged brain, which is associated with several of the growth factor genes found to be differentially expressed. Thus, the findings within this thesis are suggestive of alterations to endothelial function and the MAP-kinase pathway (Figure 7.1) and also many other pathways, which are associated with increased expression of ROS. Furthermore, the gene expression study yielded evidence of alterations to the Insulin growth factor binding protein gene, which can be associated with alterations in the MAP-kinase pathway and can impact on WM integrity. This may represent evidence of a target gene, which shows altered expression prior to overt hypertensive induced structural alterations and could be monitored in humans to further investigate its role. Moreover, the present study identified a vascular specific and global inflammatory response to hypertension. Evidence of this inflammatory response was found after alterations to eNOS expression in the young brain suggestive of alterations in endothelial signalling, which although not tested could lead to an altered endothelial function, which in turn may have caused an increased inflammatory response. Markedly this increased vascular response can also induce alterations to Insulin growth factor binding protein and components of the MAP-kinase pathway. Furthermore the evidence of altered BBB structure, endothelial signalling and microglial recruitment is indicative of possible alterations to ion channels, as found in differential gene expression. Therefore, by studying hypertension in a sustained, controlled manner the present study has isolated initial mechanisms, which are altered with hypertension. Additionally the structural alterations found were overall

subtle and in absence of a functional cognitive impairment, thus providing an ideal therapeutic window for the treatment of hypertensive related cerebrovascular pathology prior to a marked functional impairment.

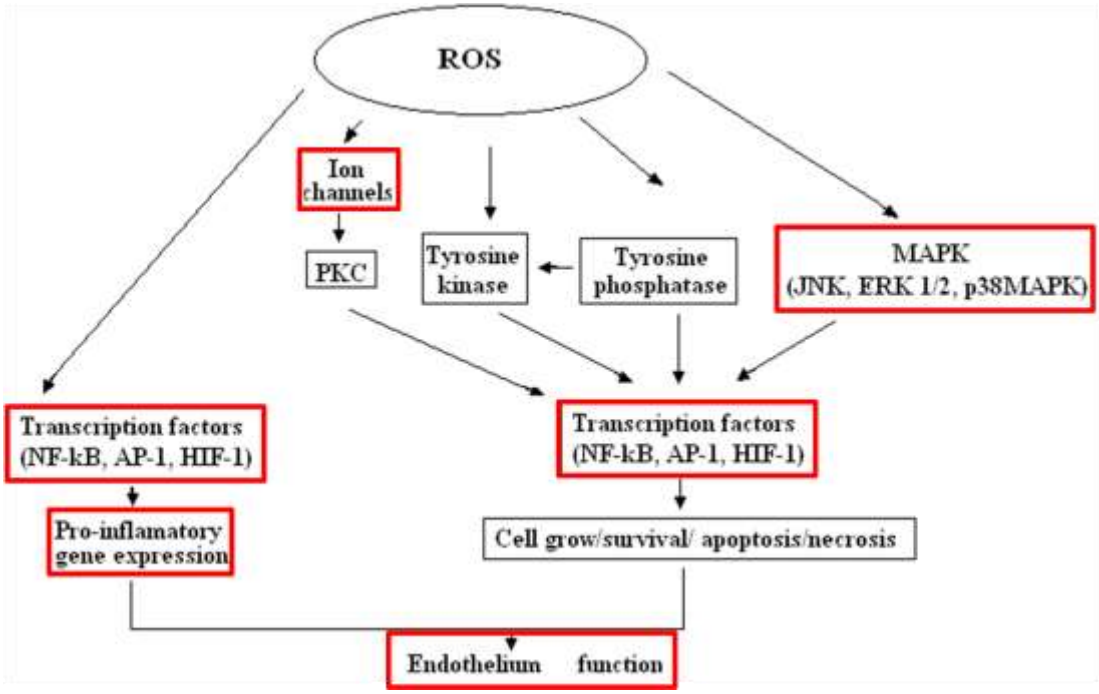

Figure 7.1: Major pathways activated by the generation of reactive oxygen species (ROS) and those found to be activated by hypertension

In the introduction, it was described that hypertension was thought to cause alterations to ROS expression leading to the activation of the above pathways, which can influence endothelial function by promoting or hindering vasodilation and activate pro-inflammatory state. Those marked in red represent the pathways found to be altered due to hypertension within this thesis (Image adapted from (Nina and Elena, 2012).

7.2. Hypertension leads to modest structural alterations to the integrity of the cerebrovasculature and white matter in the brain of the Cyp1a1 Ren2 inducible rat model

In the Cyp1a1 Ren2 inducible rat model, hypertension was induced in a gradual, controlled and sustained manner, in an aid to replicate hypertension in humans and to avoid modelling malignant hypertension. The controlled modelling of hypertension allowed clarity within the literature establishing hypertension in isolation leading to modest structural alterations to the integrity of the brain. However, this model did not examine all features of hypertension in humans, as more recent studies have provided evidence that blood pressure variability is strongly associated with WML (Havlik et al., 2002). This has lead to studies examining ambulatory blood pressure (daytime, night-time and 24 hour blood pressure averages) and correlating the variability of blood pressure throughout the day with the presence and severity of WML.

In humans, blood pressure is altered with normal daily activity which can be exacerbated with hypertension (Staessen et al., 2003), especially in those with cerebrovascular pathology (Heckbert et al., 1997). The relationship between high and low blood pressure has been observed in a longitudinal imaging study, which found a U-shaped or J-shaped relationship existed between blood pressure and the formation of WML (De Leeuw et al., 1999). This study provided evidence that periods of low and high blood pressure are both equally harmful to the brain. Data from the Honolulu-Asia aging study examined the relationship between blood pressure variation in midlife and the late-life presence of WML. This study provided a stronger association between blood pressure variation and the formation of WML,

versus controlled blood pressure and proposed that periods of high and low blood pressure may overcome autoregulation exposing the brain to periods of insufficient blood flow (Havlik et al., 2002). The variability of blood pressure and its effect on the brain has been examined by correlating periods of high blood pressure at specific times of the day, allowing the presence and severity of WML to be associated with individual blood pressure profiles. These studies have provided evidence of increased blood pressure at specific periods of the day (rather than increased blood pressure throughout the day) and this correlates more strongly with the number of lacunes and WML (Goldstein et al., 1998; Shimada et al., 1990).

These fluctuations in blood pressure can become specifically harmful in elderly treated and untreated hypertensive patients which have vascular remodelling leading to impaired autoregulation. These vascular alterations require a higher threshold of blood pressure to maintain sufficient blood flow (Heckbert et al., 1997) and can lead to periods of nocturnal hypotension. On this basis, studies have examined WML in the context of dippers i.e., patients whose blood pressure drops to hypotensive levels and non-dippers. It has been shown that there is an increased severity and volume of WML and lacunes in hypertensive patients who are extreme dippers and those who are non-dippers; increased nocturnal blood pressure, compared to patients whose blood pressure is decreased by a normal but not hypotensive nocturnal level (Kario et al., 1996; Shimada et al., 1992). In turn, ambulatory blood pressure monitoring is a useful diagnostic tool avoiding misdiagnosis and is gradually being introduced within NHS guidelines (Ritchie et al., 2011). There is a percentage of the population referred to as ‘white coat hypertensives’ that experience transient increased blood pressure during blood

pressure measurements (Pickering Tg, 1988). Ambulatory monitoring would prevent these individuals from being exposed to hypotension by unnecessary blood pressure medication.

7.2.1. *Further studies to examine cerebrovascular and white matter integrity in the Cyp1a1 Ren-2 rat model*

Although the present study within this thesis chose to examine the effect of hypertension in a controlled manner this model could be used to examine the effect of blood pressure variation. Previous characterisation studies in this model describe hypertension induction by dietary induction or gastric gavage (Kantachuvesiri et al., 2001). Both methods could be chosen to induce periods of severe hypertension. Increasing the concentration of I3C through dietary induction would allow a stress free increase in blood pressure; although blood pressure would take 48 hours to rise and would be increased until I3C concentration was lowered. Gastric gavage in combination with dietary induction would allow for increased blood pressure at specific periods of the day. These methods of blood pressure variation could be evoked with constant blood pressure monitoring by telemetry measurements. In addition the advantage of this model is that blood pressure is fully reversible after the removal of I3C. This would allow the establishment of repair mechanism and at the time point at which pathology is reversible with blood pressure lowering. This would be of great clinical importance as studies examining treatment of hypertension and reversibility of brain morphology, finding that not all alterations are reversible (Nobili et al., 1993; Raz and Rodrigue, 2006; van Dijk et al., 2004). Therefore, modelling hypertension in a reversible model would allow the opportune period for antihypertensive treatment to be established. In turn, knowing the severity of

hypertension which leads to irreversible cerebrovascular alterations would allow treatment to be adjusted to prevent periods of hypoperfusion.

7.3. Hypertension leads to a marked inflammatory response in the brain of the young and aged Cyp1a1 Ren2 inducible rat model

Even though findings within this thesis provided evidence of minimal myelin and cerebrovascular alterations with hypertension the most robust finding was the exacerbated inflammatory response. The inflammatory response was measured by the increased presence of microglial cells within specific regions as a whole and their association with the vascular. In humans there is evidence to suggest that inflammation plays a role in the mechanisms of hypertensive pathology. Cross-sectional studies have found increased serum levels of multiple inflammatory markers which are associated with microglia such as C-reactive protein, TNF-α, interleukin 6, monocyte chemoattractant protein 1 and ICAM-1 in hypertensive patients and have been shown to decrease in some patients with antihypertensive treatment (Cachofeiro et al., 2009; Fliser et al., 2004; Koh et al., 2003; Rahman et al., 2002; Sanz-Rosa et al., 2005). In turn, studies have also shown an association with increased inflammatory markers and the presence of WML (Fornage et al., 2008) and cerebral atrophy (Baune et al., 2009) in the aged brain. Studies in experimental models of hypertension have shown that antihypertensive treatment lowers levels of inflammatory mediators comparable to controls and reduces vascular remodelling (Ando et al., 2004). Therefore, the importance of the findings within this thesis was the inflammatory response in the young brain found after alterations in endothelial signalling.

7.3.1 Further studies examining the inflammatory response in the Cyp1a1 Ren-2 rat model

As described above the findings and observations in previous studies associate an influential role of microglia and hypertensive induced pathology but the mechanisms are not yet defined. Since antihypertensive and other vascular risk factors may affect the inflammatory response of hypertension in man, to study hypertension in isolation future studies would need to be carried out in animal models. The Cyp1a1 Ren2 rat model could provide a tool to examine the role of microglia in relation to vascular remodelling using an in-vivo imaging approach as described in a previous study (Davalos et al., 2005). This could have been assessed by creating Cyp1a1 Ren2 inducible transgenic rats with GFP tagged microglia and vasculature visualised using in-vivo two photon laser capture imaging. This would allow the response of microglia to the surrounding environment with hypertension and in relation to vascular alterations to be examined. Additionally, the animals could be given an anti-inflammatory drug and the response of the microglia in relation to the vascular alterations could be examined.

However, as discussed previously and of great importance the method of hypertension induction in this model by I3C, may have lowered the inflammatory response, thus this study would also need to examine non-transgenic Fischer animals under normal conditions and inflammatory induced conditions to investigate the impact of dietary indole-3-carbinol on the inflammatory response.

In turn, the vascular integrity with hypertension alone and hypertension with anti-hypertensive treatment could be further investigated at a higher resolution using contrast-enhanced magnetic resonance microangiography (CE-μMRA) as described

by Klohs et al., 2012. This form of imaging would allow a three dimensional assessment of vascular structure in-vivo and assessment of the number of functional vessels within the substance of the brain, which could be correlated to the previous microglia assessment study. These studies could also be run in parallel with previously suggested studies to investigate varying severities of hypertension on vascular integrity and microglia response.

7.4. Initial mechanisms of hypertension leads to alterations in endothelial signalling in the Cyp1a1 Ren2 inducible rat model

Within this thesis data provided evidence of alterations to endothelial signalling prior to vascular remodelling and inflammatory response suggestive of an initial response of the vasculature to hypertension. Evidence of alterations in endothelial signalling in particular decreased NO bioavailability has been reported with hypertension in humans and experimental models (Briones and Touyz, 2010). The level of endothelial dysfunction may be exacerbated in this study as mature endothelial cells have limited regenerative capacity and endothelial progenitor cells have been shown to be altered in migratory ability and function with hypertension (Imanishi et al., 2005). In turn, as discussed previously endothelial dysfunction is associated with increased endothelial progenitor cells, ROS, vasoconstrictive agents and decreased NO availability (Giannotti et al., ; Vanhoutte, 2009; Yao et al., 2006). Based on previous literature and data provided within this thesis is suggestive that early alterations in endothelial signalling progress to vascular remodelling.

7.4.1 Further studies examining endothelial signalling in the Cyp1a1 Ren2 inducible rat model

Further studies could be carried out to investigate whether alterations in the number of endothelial progenitor cells contribute to alterations in eNOS expression in the Cyp1a1 Ren2 rat model, by fluorescent labelling of endothelial progenitor cells with a proliferative marker. This study could examine eNOS levels at earlier time points than those investigated in this thesis and with addition of an endothelial progenitor marker to establish the initiation of alterations to endothelial signalling. In addition, by reversal of hypertension in this model the onset and reversibility of endothelial dysfunction could be established. It is accepted in the majority of the literature that endothelial dysfunction is an early feature of hypertension, however the recommended first stage antihypertensive treatments do not directly address this (Krause et al., 2011). Statins directly influence endothelial function through ENOS (Endres et al., 2004). However, under NHS guidelines these are prescribed to patients as a mode of secondary protection in response to ischemic heart disease or peripheral vascular disease i.e., angina or previous myocardial infarction (Krause et al., 2011; Network, 2012). Therefore based on data within this thesis prescribing therapeutic agents to improve eNOS production at this stage may increase circulating ROS and further exacerbate the inflammatory response. Therapeutic interventions aimed at improving endothelial function may be more beneficial prior to vascular remodelling and also concomitantly with an anti-inflammatory agents to prevent any secondary effects of increased ROS production.

7.5 Overall limitations of the thesis

Although the data containing within this thesis provides evidence of initial subtle structural alterations caused by hypertension in isolation, it does not fully investigate the functional impact of these alterations. Since there is evidence of alterations to the cerebrovascular structure in the young 6-month cohort, it would be interesting to analyse what impact these have on blood flow, vessel contractility and also vessel leakiness. These functional implications could be anaylsed using laser Doppler to investigate blood flow in combination with pharmacological stimulation to evoke different pressure values, wire myography to investigate the ability of the vasculature to constrict and dilate in response to pharmacological stimuli and vessel leakiness could be examined by horse radish peroxidase injections combined with immunohistochemistry.

Additionally it would be interesting to underpin the indirect functional alterations cause by these structural alterations to the cerebrovasculature. Within this thesis cognitive function in the form of spatial reference and working memory was assessed in the young brain when initial but relatively subtle structural alterations were found. Although this study found no evidence of alterations to spatial reference or working memory, it would be useful to examine the impact of the structural alterations found on neuronal connectivity, at each time point. This could be achieved by the use of electrophysiology under normal brain function and periods of induced hypercapnia.

7.6 Future direction within the field of hypertension in general

The studies carried out within this thesis choose to examine hypertension using the Cyp1a1 Ren2 inducible rat model, for reasons already described, but the SHR and SHRSP models have undoubtedly provided a wealth of information to the field. Even though there are issues concerning appropriate normotensive control comparisons within these models, it would be interesting to carry out studies comparing the three models as each invokes different forms of hypertension. It would also lean towards strengthening specific hypertensive related findings if they were present in each model.

Animal models are frequently used to study hypertension due to its coexistence with several vascular risk factors in humans. However, within the field it is necessary that we grasp what impact the con-existence of these vascular factors has on the structural and functional alterations observed in the aging human brain. It is unclear if it is solely the presence of multiple vascular factors or if it is a specific combination of vascular risk factors, which are particularly detrimental to the structural and functional integrity of the brain. Thus, further studies are required, which use a combination of vascular risk factors to assess their impact on the structural and functional integrity of the brain.

Last but certainly not least, as introduced within the beginning of this discussion blood pressure in humans fluctuates temporarily throughout the day but patients are prescribed pharmaceutical methods designed to constantly lower blood pressure in such that they may be prone to periods of hypoperfusion. This needs to be carefully assessed as to the impact on the structure and function of the brain. This

may require the development of antihypertensive treatment, targeting populations with vascular remodelling, which may be designed with a shortened half-life in an aid to prevent blood pressure lowering actions during periods when the patient's blood pressure naturally dips.

7.6. Concluding remarks

Based on evidence provided within this thesis, hypertension induced in a controlled and sustained manner leads to modest alterations to the integrity of cerebrovasculature structure and WM. This study highlighted initial endothelial dysfunction caused by hypertension, which predisposed the brain to an exacerbated inflammatory response and vascular remodelling. Further work, using this inducible model of hypertension could help to unravelling the mechanisms leading to alterations in endothelial signalling and fully establish the link between endothelial function, vascular remodelling and inflammation. Work such as this would impact positively on the understanding of hypertension as a risk factor for cerebrovascular disease and age related cognitive decline but also in therapeutic approaches preventing the impact of hypertension on the brain.

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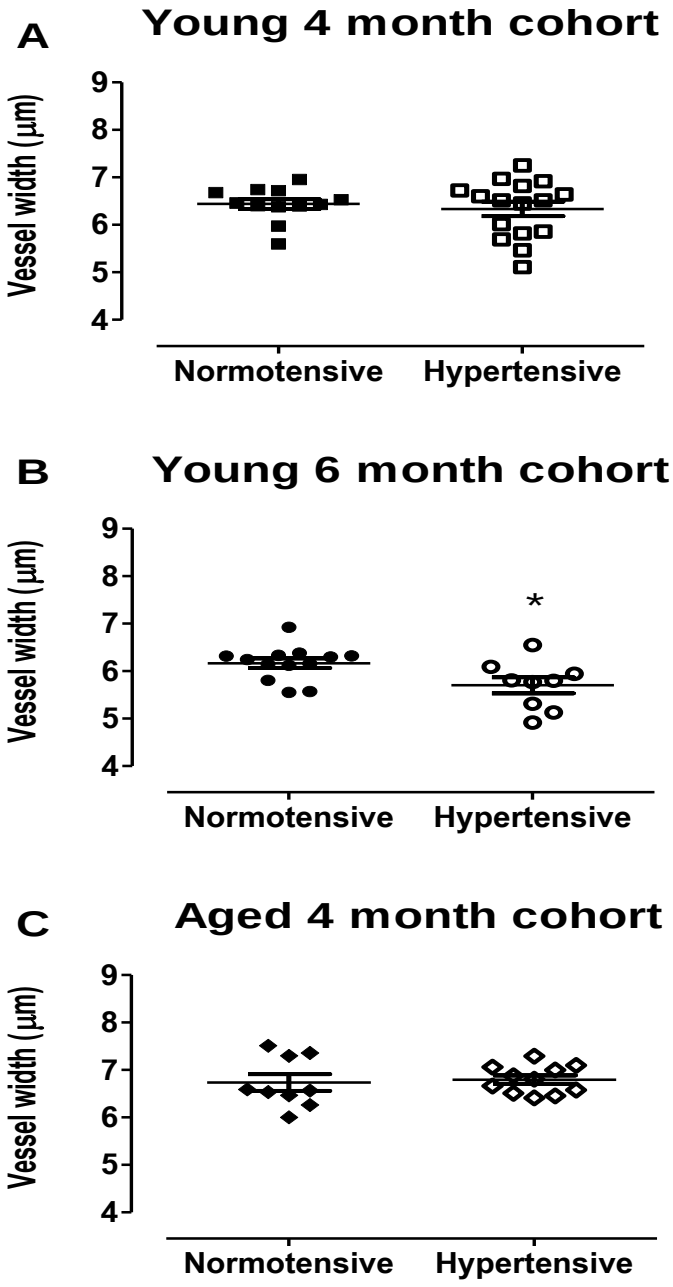
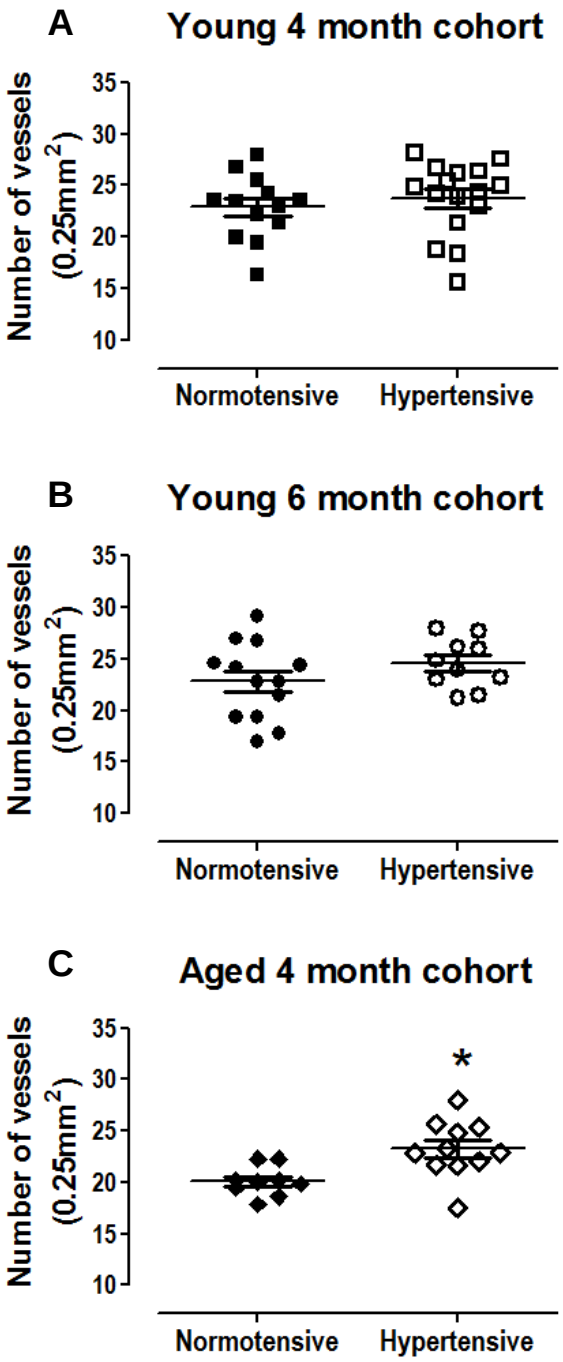
Appendix A: Additional vascular data



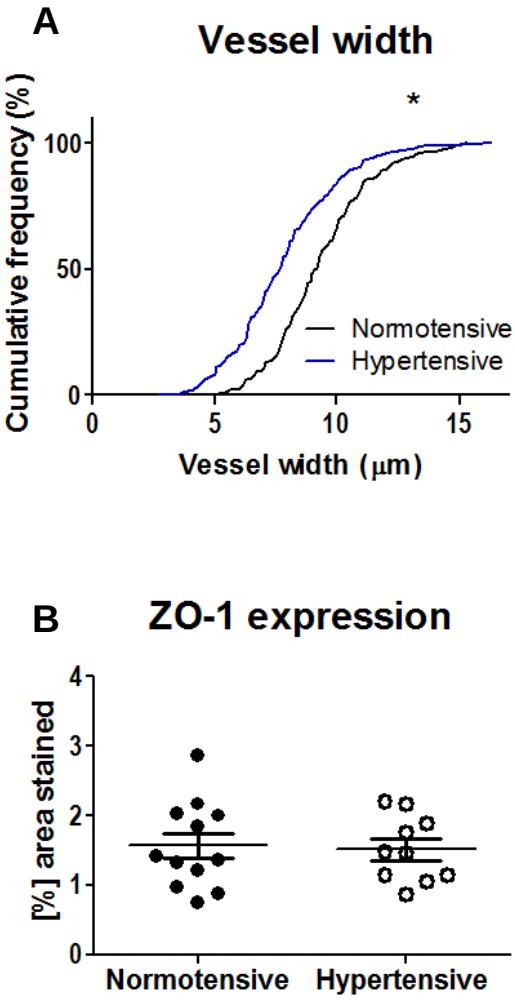
A1: Vessel width

Collagen IV vessel width data was presented within the thesis as frequency distribution. The above graths show the averge vessel width measurement per animal in the (A) young 4 –month cohort, (B) young 6- month cohort and (C) aged 4 month cohort.



A2: Number of vessels

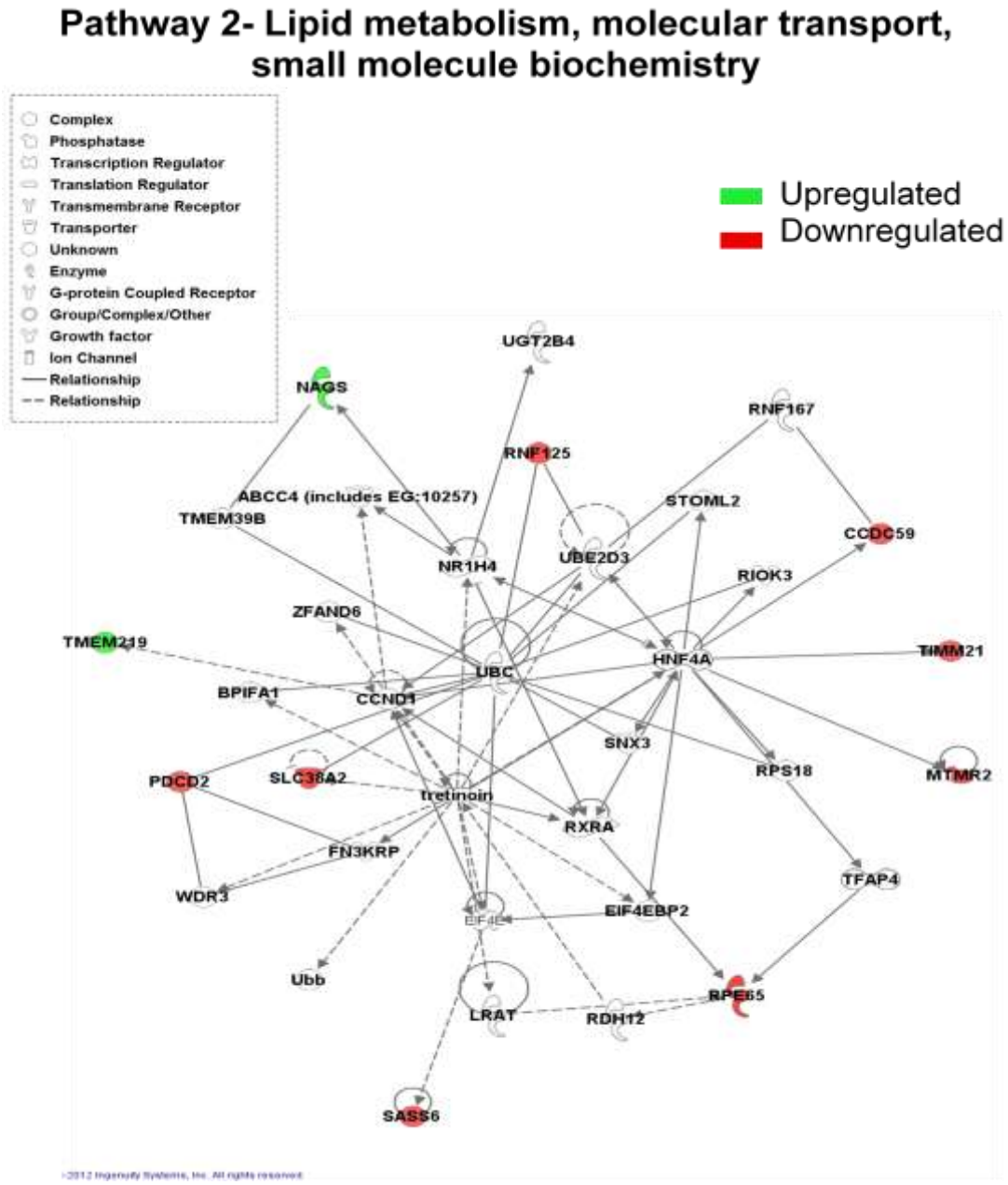
There was no significant difference in the number of vessels visualised using antibody specific for Collagen IV, in hypertensive animals when compared to normotensive from the young cohorts (A-B). However there was a significant increased in the number of vessels in hypertensive animals from the aged 4-month cohort when compared to normotensive (C). Significance *$p<0.01$.



A3; Addition tight junction data from the young 6-month cohort

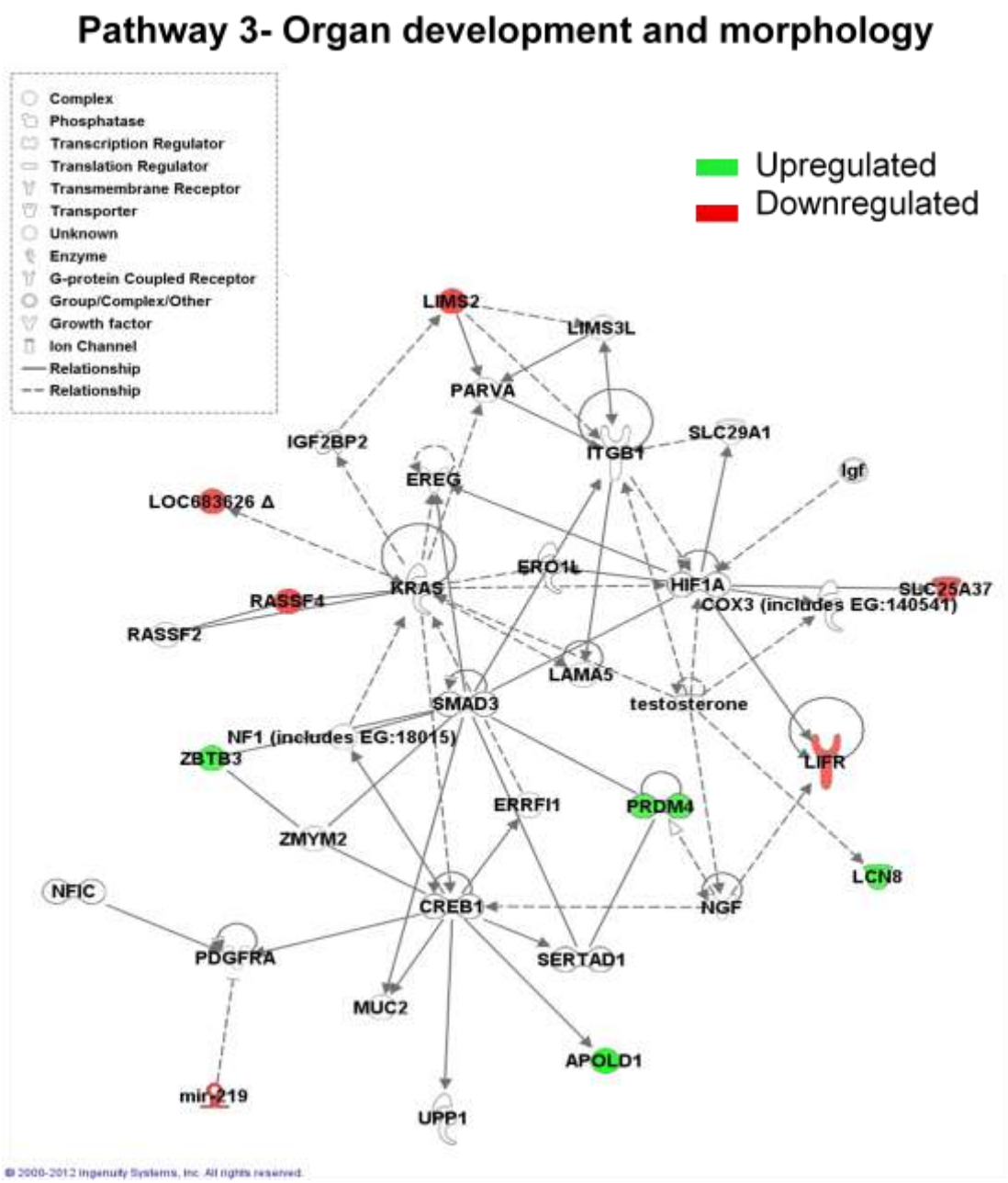
(A), vascular width measurement analysed by Claudin-5 staining providing evidence that hypertension leads to significantly smaller vessels, analysed by frequency distribution. (B) No alteration in the density of ZO-1 staining in hypertensive animals when compared to normotensive. *$p<0.01$.

Appendix B Additional microarray data from the young 6-month cohort



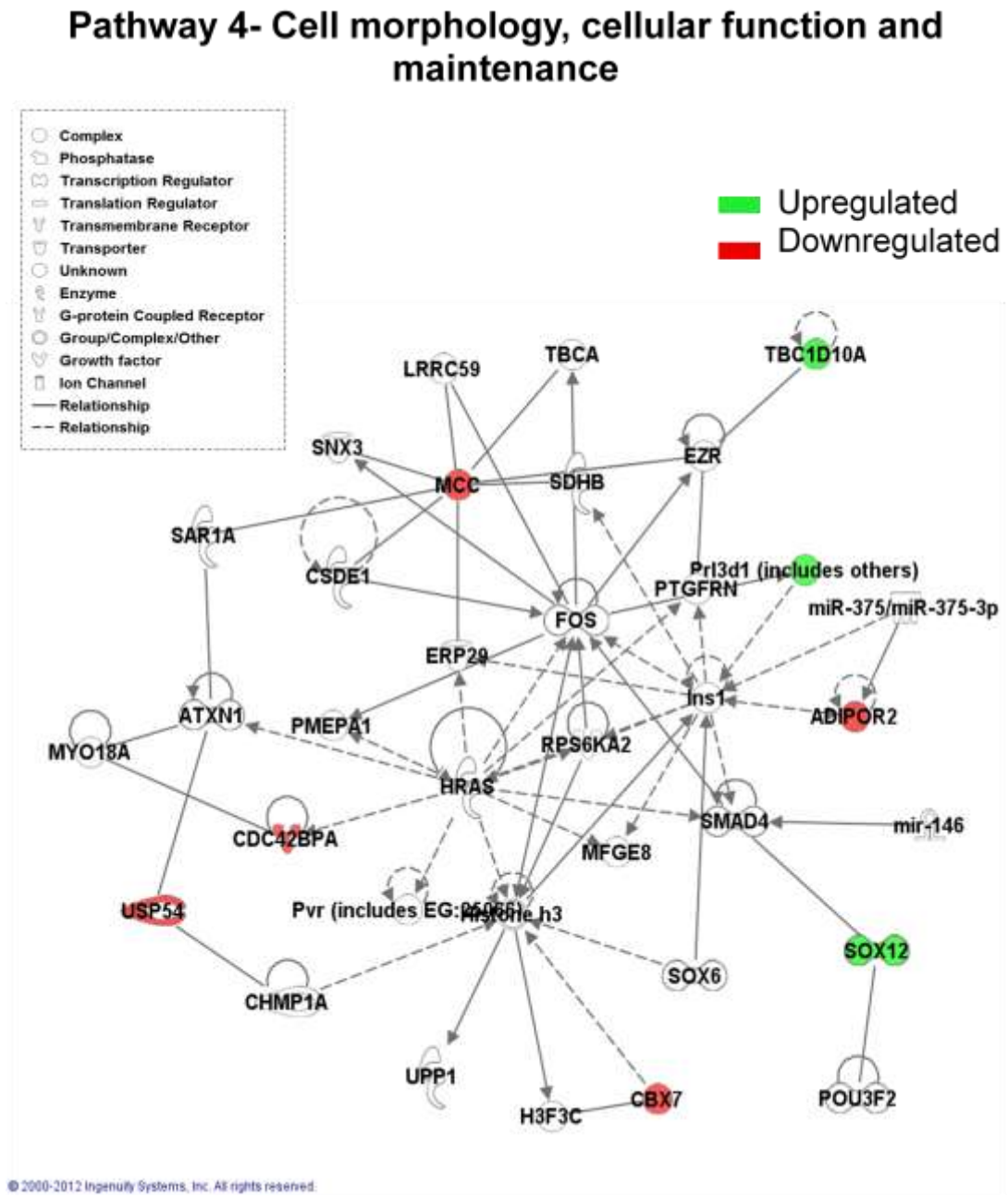
B1: Pathway 2- Lipid metabolism, molecular transport, small molecule biochemistry

Upregulated genes are in green and downregulated genes are in red in hypertensive animas versus normotensive. Within this pathway hypertension leads to differential gene expression of genes related to energy metabolism and a few inflammatory related genes.



B2: Pathway 3- Organ development and morphology

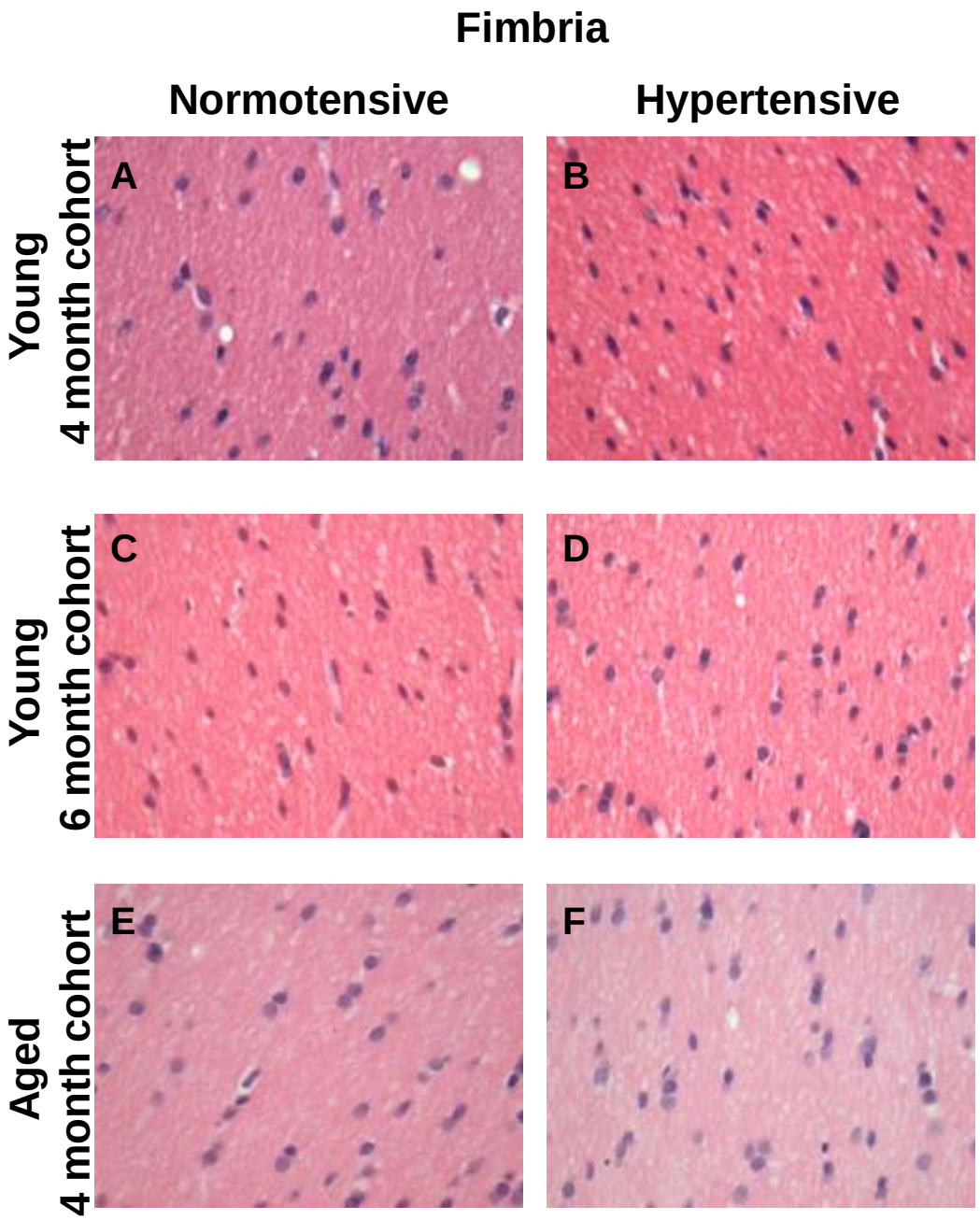
Upregulated genes are in green and downregulated genes are in red in hypertensive animas versus normotensive. Within this pathway hypertension leads to differential gene expression of small molecule transport and inflammatory genes.



B3: Pathway 4- Cell morphology, cellular function and maintenance

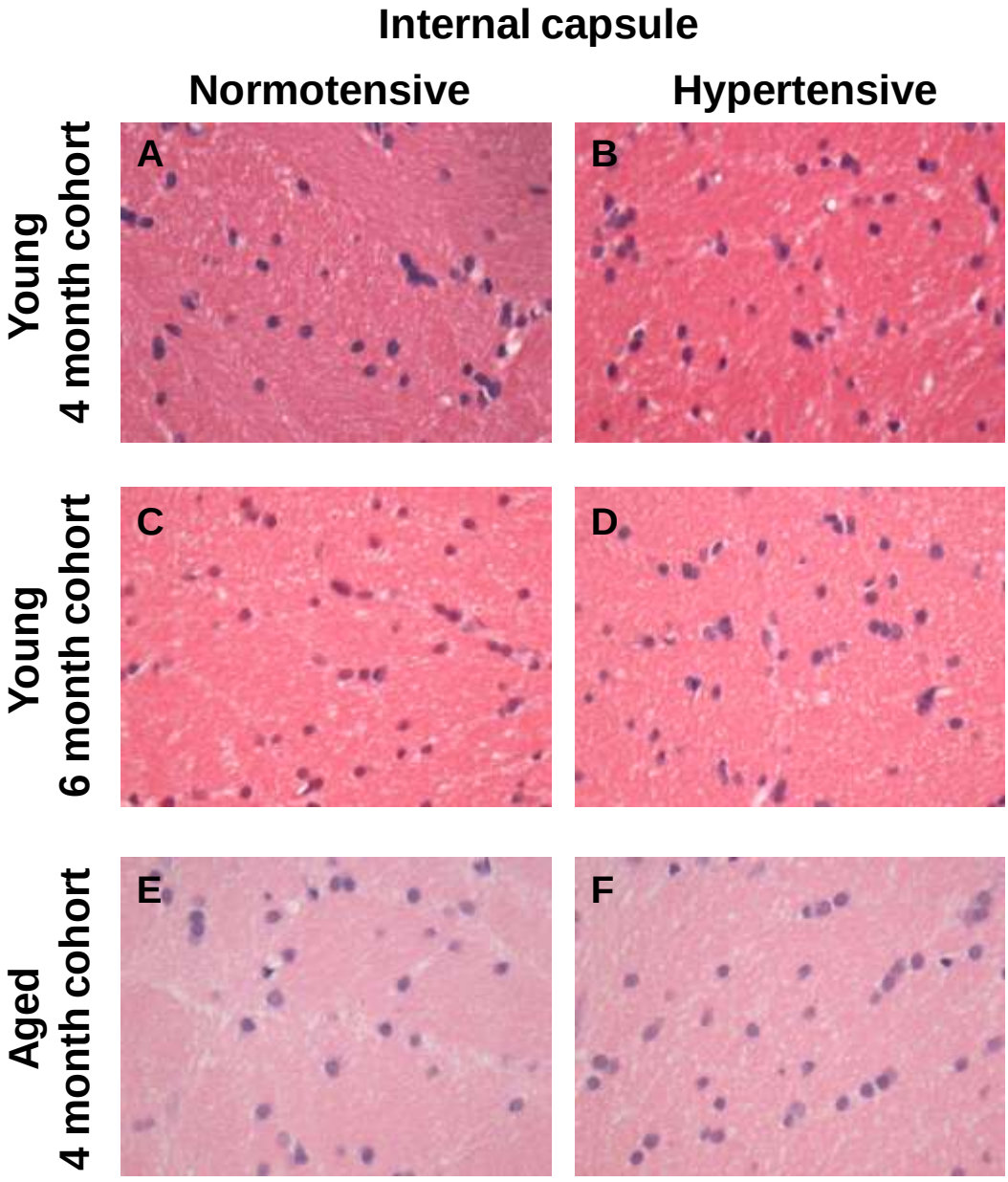
Upregulated genes are in green and downregulated genes are in red in hypertensive animas versus normotensive. The last pathway is the least significant pathway, with fewer genes and the majority of which function is unknown.

Appendix C Additional H&E images representing the structural integrity of white matter



C**1: Representative images of no overt structural alterations in the fimbria.**

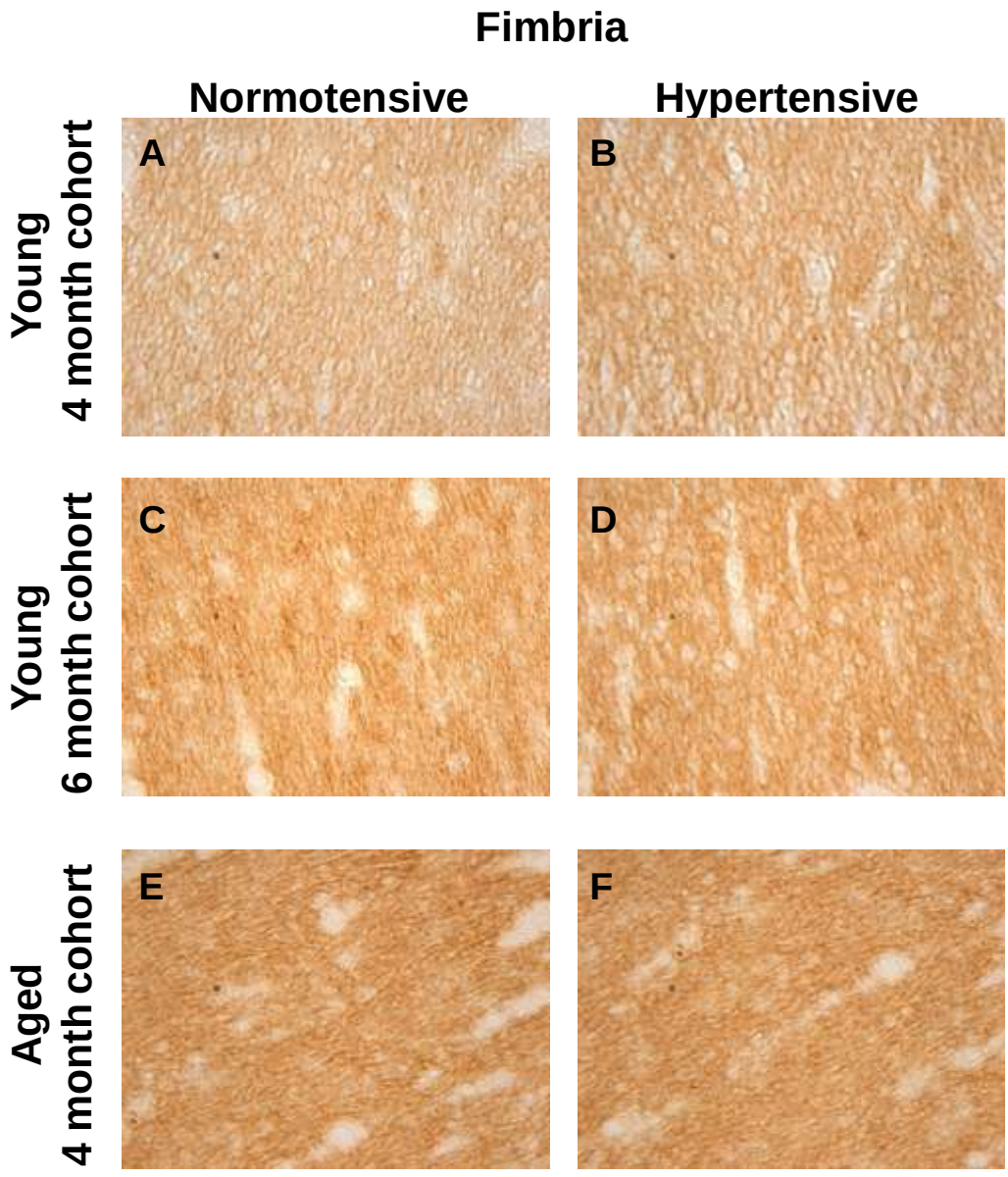
H&E was used to analyse the structural integrity of the fimbria in each cohort. No evidence of tissue pallor, microbleeds or haemorrhages were found in normotensive (A, C, E) or hypertensive (B, D, F) animals.



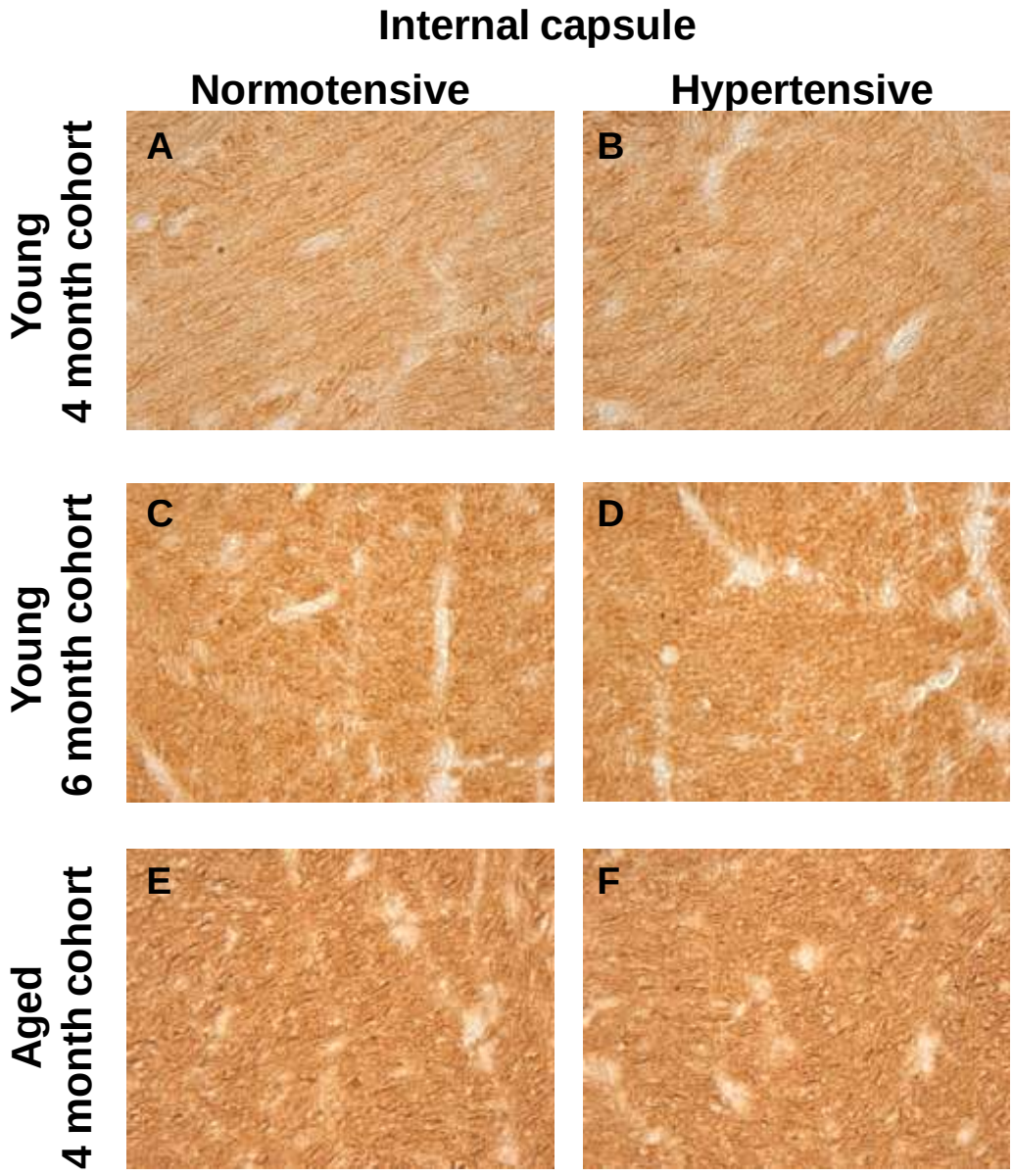
C2: Representative images of no overt structural alterations in the internal capsule.

H&E was used to analyse the structural integrity of the internal capsule in each cohort. No evidence of tissue pallor, microbleeds or haemorrhages were found in normotensive (A, C, E) or hypertensive (B, D, F) animals.

Appendix D Additional representative images of MBP staining in the white matter

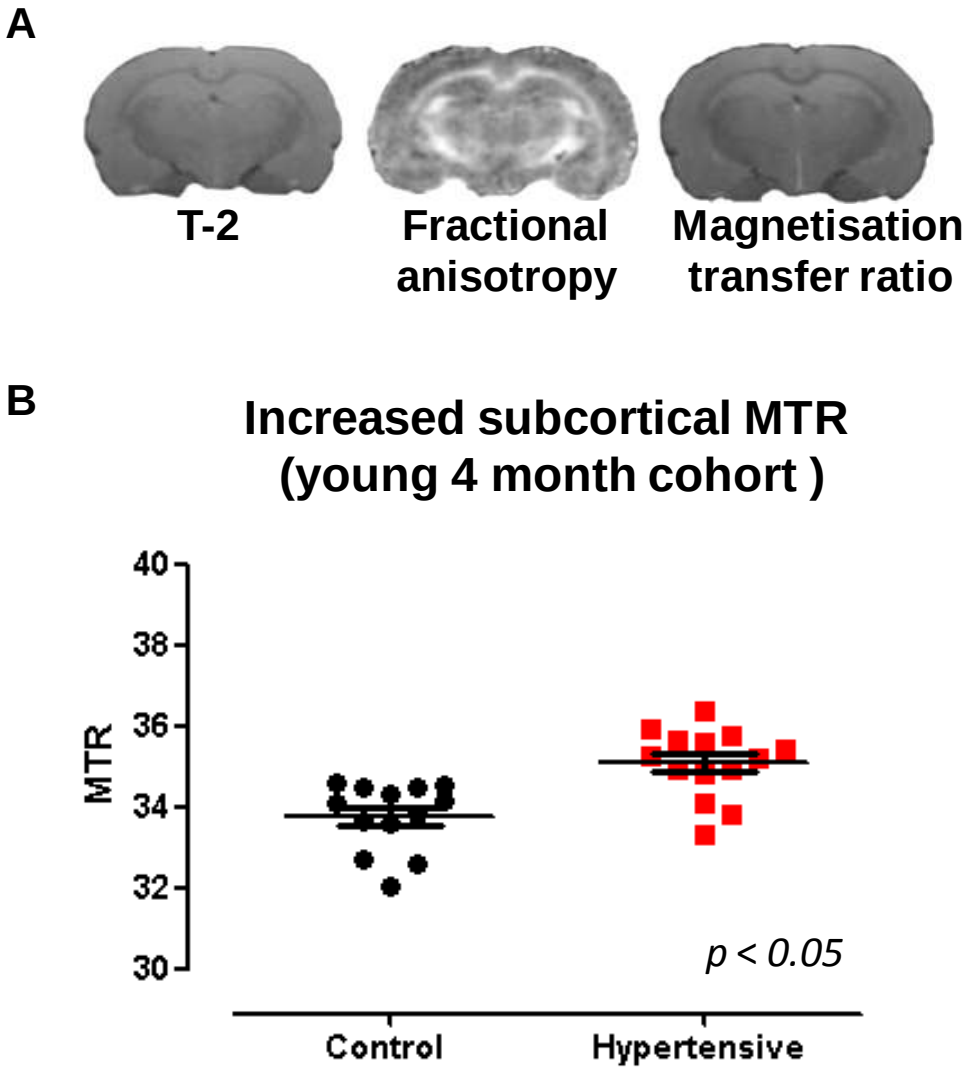


D1: Representative images of MBP staining in the fimbria.
There was no evidence of alterations in levels of myelin visualised using MBP within the Fimbria of hypertensive animals (B, D, F) compared to normotensive (A, C, E) from all three cohorts.



D2: Representative images of MBP staining in the Internal capsule.
There was no evidence of alterations in levels of myelin visualised using MBP within the internal capsule of hypertensive animals (B, D, F) compared to normotensive (A, C, E) from all three cohorts.

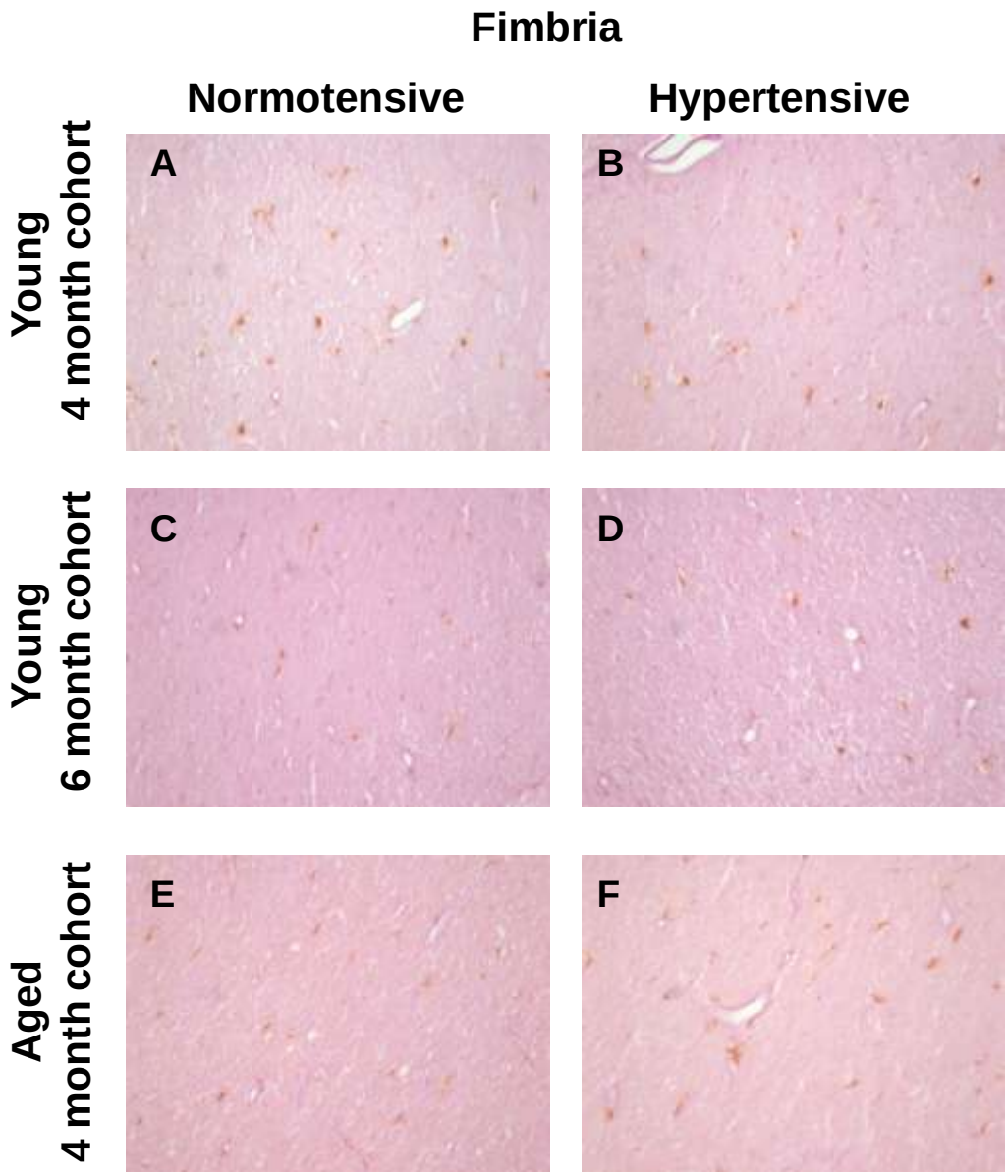
Appendix E Additional data of Magnetic resonance imaging findings in the young 4-month cohort



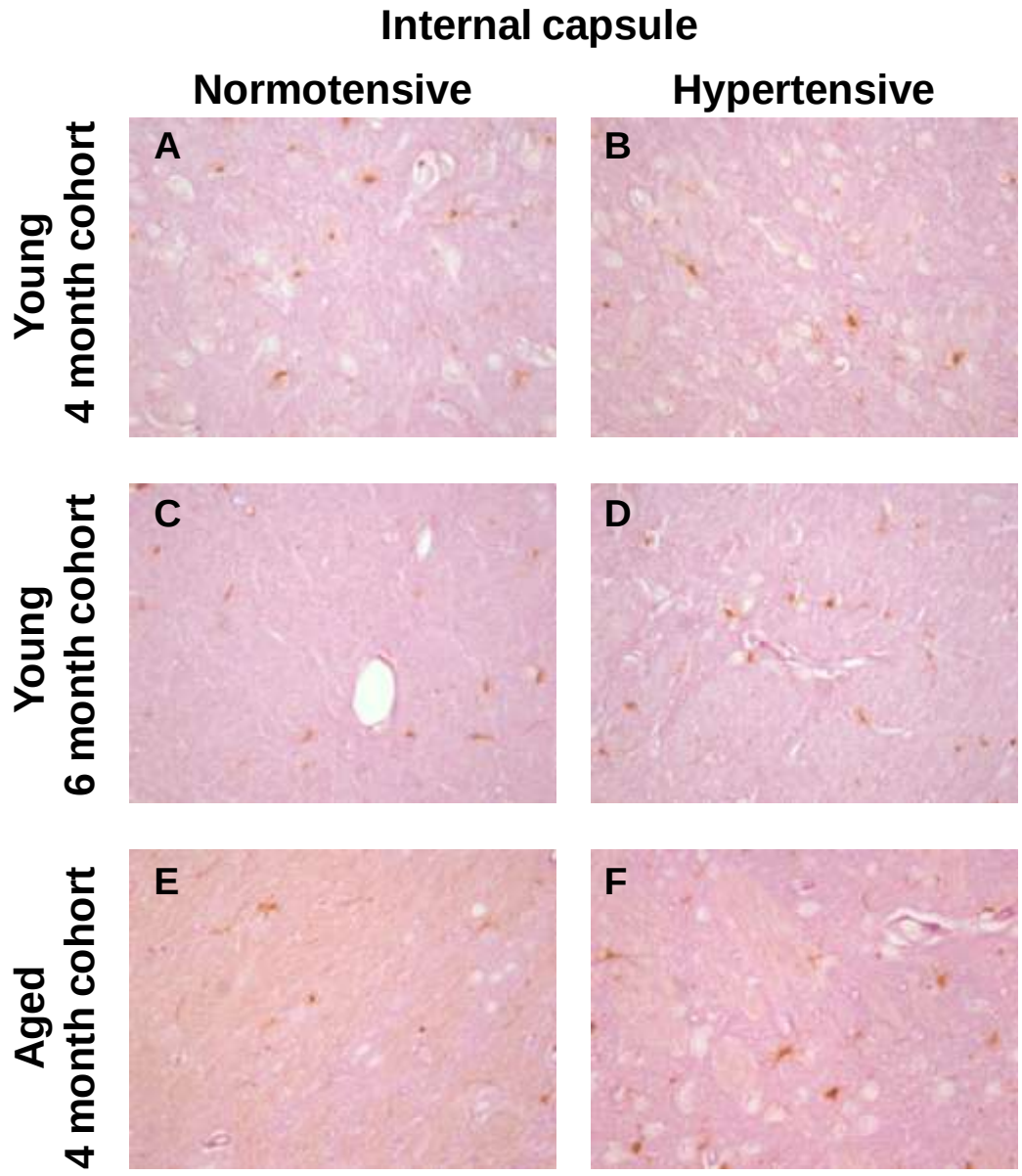
E1: MRI in the young 4-month cohort

MRI investigated of the stucutral integrity of the brain with hypertension (A). In young 4-month hypertensive animals there was a significant increase in magnetisation transfer ratio (MTR), when compared to normotensive controls (B). *$p<0.05$

Appendix F Additional representative images of microglial expression in the white matter

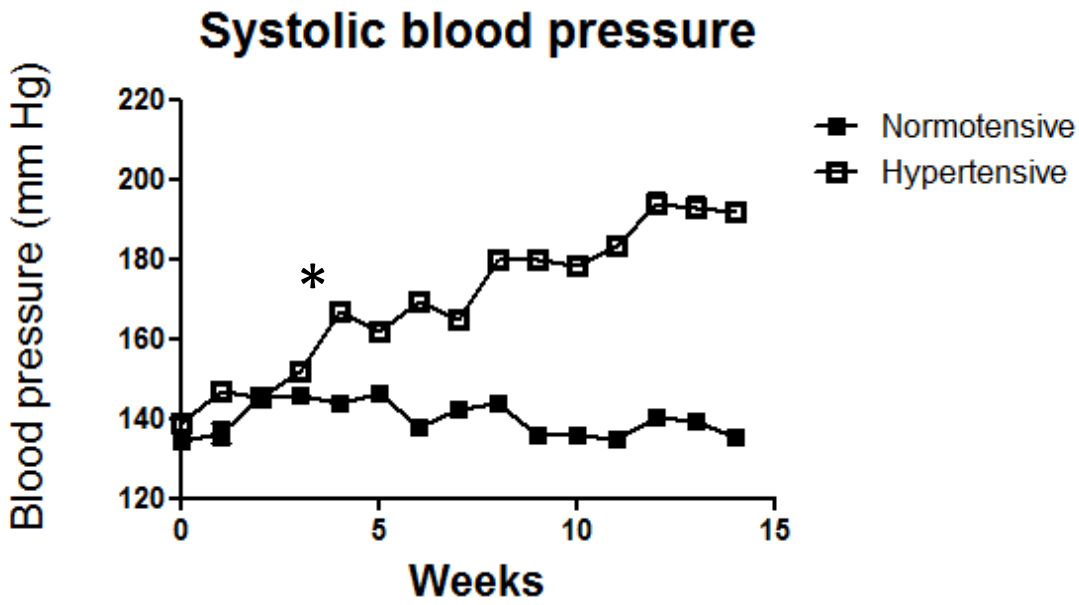


F1: Representative images of microglia expression in the fimbria.
There was no evidence of alterations in numbers of microglia, visualised using IBA1 (brown), within the Fimbria in hypertensive animals (B, D, F) compared to normotensive (A, C, E) from all three cohorts.



F2: Representative images of microglia expression in the fimbria.
No evidence of alterations in the numbers of microglia, visualised using IBA1 (brown) within the Internal capsule in hypertensive animals (B) compared to normotensive (A) from the young 4-month cohort. However, there was a significant increase in the number of microglia in hypertensive animals from the young 6-month cohort (D) and the aged 4-month cohort (F) when compared to normotensive (C, E).

Appendix G Additional blood pressure data from the young 4-month behaviour cohort



G1: Behaviour cohort systolic blood pressure measurements

Similar to previous studies animals fed the I3C hypertensive diet have significantly increased systolic blood pressure. Anaysed by repeated measures ANOVA as previously found there is a significant difference in blood pressure over time (p<0.001) and a significant interatction between blood pressure and treatment in hypertensive versus normotensive animals (p<0.001) and post hoc t-tests show a significant diffenrence at week 5 post dietary induction when compared to animals fed a normotensive diet of no I3C. *p<0.01.